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Fatty acids and lipid signaling in grapevine resistance to *Plasmopara viticola*

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Em cumprimento com o disposto no referido despacho, esclarece-se ser da minha responsabilidade a execução das experiências que estiverem na base dos resultados apresentados (exceto quando referido em contrário), assim como a interpretação e discussão dos mesmos.

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Abstract

Grapevine (*Vitis vinifera* L.) is one of the most cultivated crops in the world, with over 7.5 million production hectares. Among the known 70 *Vitis* species, *Vitis vinifera* is the only used for wine production, generating revenues over 29 billion euro in 2016. However, this *Vitis* species is highly susceptible to several fungal diseases, namely to downy mildew. Downy mildew is caused by the biotrophic obligatory oomycete, *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni. It was introduced in Europe in the late 19th century and soon became one of the most economically significant grapevine diseases worldwide. Current strategies to cope with this disease rely on the preventive use of phytochemical compounds on each cultivation season, representing high environmental, economical and health costs. Thus, characterization of innate resistance of some *Vitis* species and *V. vinifera* genotypes to *P. viticola* is crucial for the definition of sustainable disease control measures. Previous studies have shown that membrane lipids present not only a structural function but also play an important role in plant defense, providing substrates for signaling molecules, such as free fatty acids (free FA), phosphatidic acid (PA), inositol 3 phosphate (IP_3), oxylipins and others. The release of fatty acids (FA) from membrane lipids is carried out by phospholipases that are activated upon pathogen perception. Among phospholipases, the role of phospholipase A (PLA) is highlighted by its participation in plant immunity, mainly through its role in jasmonic acid (JA) biosynthesis.

In the present work we have characterize the FA and lipid modulation in two *Vitis vinifera* genotypes resistant and susceptible to *P. viticola*. Our results provide new insights on the modification of lipid FA profile in the first hours after pathogen challenge. Major FA modulation seems to occur in the resistant grapevine genotype Regent, α-linolenic acid (C18:3) content increases in all of the studied time-points as well as the ratio between unsaturated and saturated FA and membrane fluidity. Being 6 hours post-inoculation (hpi) the time-point were major FA alterations occur, we have further evaluated changes in lipid classes by thin layer chromatography (TLC). In the resistant genotype the content of both phosphatidylcholine (PC), phosphatidylethanolamine (PE) decreases 6h after pathogen challenge and at the same time Mono and Di – galactosyldiacylglycerols (MGDG and DGDG) and phosphatidylglycerol (PG) content increased, particularly in the C18:3 content. Moreover, we have characterized grapevine PLA superfamily, identifying 41 PLA genes. Three major classes were defined according to PLA sequence homology: phospholipase A1 (PLA_1), secretory phospholipase A_2 ($sPLA_2$) and patatin-like phospholipase A (pPLA). A set of grapevine PLA genes was selected for gene expression analysis, in way to elucidate their role in grapevine resistance against *P. viticola*. Our results have shown that an increase of expression occurs in the majority of the selected genes, mainly at 6 hpi. Altogether our results, combined with the results previously obtained on reactive oxygen species (ROS) production and lipid peroxidation, may suggest that the decrease of PC and PE lipids content may be related to ROS signalling and PLA genes activation by Ca^{2+}. Membrane lipid hydrolysis by PLA may, at later infection time-points, contribute to the decrease of C18:3 from galactolipids and increase of free- C18:3 enriched FA thus promoting JA biosynthesis. From our previous work, we have shown that JA and JA-isoleucine (JA-ILE) content is significantly altered in Regent at 12 hpi, thus further work has to be developed in order to fully characterize the lipid-associated signalling pathways leading to JA-signalling and plant resistance.

As our results allowed also to discriminate the resistant and susceptible grapevine genotypes prior to pathogen challenge, we have also made a preliminary access of FA profile in several grapevine species and cultivars with different degrees of resistance to this pathogen. The resistant genotypes presents a distinctive lipid and FA pattern, suggesting that these molecules may represent strong candidates for the establishment of resistance associated biomarkers.

Keywords:

Vitis vinifera, Plasmopara viticola, phospholipase A, lipid signalling, jasmonic acid

Resumo alargado

A videira (*Vitis vinifera* L.) é a uma das plantas mais cultivadas em todo o mundo, com mais de 7.5 milhões de hectares de produção, a nível mundial. Foram identificadas mais de 70 espécies de *Vitis*, mas apenas a *Vitis vinifera* é utilizada para a produção de vinho, que por si só tem um mercado tão vasto que em 2016 gerou mais de 29 mil milhões de euro. Portugal é um dos maiores produtores e exportadores de vinho do mundo, posicionando-se em décimo primeiro lugar no ranking mundial dos países produtores de vinho. A cultura vinícola portuguesa é riquíssima, contando com mais de 250 variedades nacionais, o que torna a nossa indústria vinícola uma das mais conceituadas e apreciadas no mundo. Contudo a grande maioria das cultivares de *Vitis vinifera* utilizadas na indústria vinícola apresenta uma elevada suscetibilidade à doença míldio.

O míldio da videira é causado pelo *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, um oomycete obrigatório, que foi introduzido na Europa no final do século XIX, através de enxertos de videiras provenientes da América, que eram utilizados para replantar as videiras afetadas por uma praga causada por filoxera. O míldio da videira é uma das epidemias mais severas em culturas de videira, podendo numa única época devastar culturas inteiras, reduzindo a qualidade das uvas e afetando também a do vinho produzido, o que consequentemente leva a enormes perdas económicas.

O modo de infeção do *P. viticola*, requer um conjunto de condições favoráveis, como temperatura moderada (21-25ºC) e humidade elevada ($\geq$75%). Quando estas condições estão reunidas, os oósporos germinam, originado zoosporângios que libertam zoósporos que migram até ao estoma onde germinam e atravessam a cavidade estomática, de forma a desenvolver as hifas primárias e o micélio. No espaço intercelular do mesófilo ocorre a diferenciação das hifas primárias no haustório, que é uma estrutura especializada que permite que o oomycete obtenha nutrientes, suprima os mecanismos de defesa e redirecione o metabolismo do hospedeiro a seu favor. Em videiras suscetíveis, formam-se as manchas de óleo características e o desenvolvimento dos esporangióforos que após emergir através do estoma, libertam os esporângios, sendo estes dispersos até tecidos hospedeiros, como folhas, frutos ou ramos, podendo originar infeções secundárias.

De forma a controlar esta doença, os viticultores recorrem ao uso preventivo de fitoquímicos durante toda a época de cultivo, o que acarreta elevados custos económicos e ambientais. Uma abordagem alternativa, visando a sustentabilidade da viticultura passa pela criação de videiras hibridas resultantes do cruzamento de *Vitis* americanas ou asiáticas, que possuem resistência ou tolerância ao *P. viticola*, com variedades de *Vitis* com boa qualidade para a indústria vinícola. Contudo, o processo de seleção de uma videira hibrida é bastante trabalhoso e demorado, sendo necessários 2 a 3 anos até que seja possível identificar plantas que contenham as características desejadas.

O objetivo principal deste trabalho é a compreensão de como se processam os mecanismos de defesa bem como quais as alterações que as videiras resistentes apresentam no combate ao míldio. Nos últimos anos, vários estudos têm sido efetuados na área das ómicas (transcritómica, proteómica e metabolómica). Estas análises permitiram identificar diferenças entre genótipos de videira resistentes/tolerantes e suscetíveis ao *P. viticola*, nomeadamente associadas aos mecanismos de perceção do patogénio, cascatas de sinalização e sinalização por lípidos. Na sinalização por lípidos, foi demonstrada a participação do ácido jasmónico (JA) na resposta de defesa a este patogénio biotrófico e o envolvimento de lípidos membranares.

Os lípidos membranares não só têm uma função estrutural, como também estão envolvidos nos mecanismos de defesa das plantas. Em situações de stress e sob ação de fosfolipases, os lípidos de membrana fornecem substratos para a formação de moléculas de sinalização tais como os ácidos gordos (FA) livres, ácido fosfatídico, inositol trifosfato (IP_3), oxilipinas entre outros. A fosfolipase A (PLA) é um importante enzima que tem a sua atividade aumentada em situações de resposta a agentes

patogénicos, levando à hidrólise de fosfolípidos presentes nas membranas. Destas reações de hidrólise são libertadas diversas moléculas, que desempenham funções de sinalização ou servem de substratos para a biossíntese de moléculas de sinalização, como o JA.

No âmbito deste trabalho foram englobadas três vertentes, análise da modulação dos ácidos gordos e lípidos, bioinformática e transcritómica. O perfil de ácidos gordos e lípidos de dois genótipos de *Vitis vinifera* com diferente tolerância ao *P. viticola* foi caracterizado por cromatografia em camada fina (TLC) e cromatografia gasosa (GC). As cultivares de *Vitis vinifera*, Regent (resistente) e Trincadeira (suscetível) são constitutivamente diferentes, no que diz respeito à composição lipídica e de conteúdo em FA. Após inoculação com *P. viticola*, a cultivar resistente apresenta uma modelação do conteúdo em ácidos gordos não observável na cultivar suscetível, nomeadamente uma acumulação de ácido α-linolénico (C18:3) bem como um aumento do rácio de ácidos gordos insaturados e saturados, refletindo-se numa maior fluidez das membranas celulares. As maiores alterações do conteúdo em ácidos gordos ocorreram às 6h após inoculação, pelo que selecionámos este momento para uma avaliação mais detalhada da modulação das diferentes classes lipídicas. Em Regent foi observada uma diminuição dos lípidos de membrana, fosfatidilcolina (PC) e fosfatidiletanolamina (PE), dos ácidos gordos livres bem como nos lípidos de reserva, triacilglicerol (TAG). Por outro lado, os lípidos cloroplastidiais, mono e di-galactosil diacilglicerol (MGDG, DGDG) e fosfatidilglicerol (PG), aumentam com a inoculação, nomeadamente no seu conteúdo em C18:3.

As fosfolipases A hidrolisam lípidos membranares e já foram associadas à biossíntese de JA. Com o objetivo de uma melhor compreensão das vias metabólicas, associadas às alterações lipídicas observadas no genótipo resistente, sobretudo associado ao JA, focámos o nosso estudo nesta família. Conduzimos uma análise bioinformática para identificar e caracterizar os membros desta família em *V. vinifera*. Identificámos 41 genes, localizados em 9 dos 19 cromossomas da videira, que codificam proteínas que se distribuem em três grandes classes, fosfolipase A1 (PLA_1), fosfolipase A2 secretora ($sPLA_2$) e fosfolipase A de tipo patatina (pPLA), de acordo com a homologia de sequência. Alguns genes foram selecionados para uma análise de expressão por PCR em tempo real (qPCR) em *Vitis vinifera* cv. Regent inoculada com *P. viticola*. Os resultados obtidos demostraram que a maioria dos genes selecionados apresenta aumento de expressão, sobretudo nas primeiras horas de infeção. Um dos ácidos gordos libertados por estas enzimas é o C18:3, que é o precursor biossintético do JA, facto corroborado por uma acumulação significativa de JA, bem como a sua forma ativa JA-isoleucina (JA-Ile), às 12 horas após a infeção com o *P. viticola* previamente descrita para genótipo resistente, Regent.

Os nossos resultados demonstraram também que o perfil de ácidos gordos permite discriminar ambos os genótipos, Regent e Trincadeira, antes da infeção com o *P. viticola*. De forma a avaliar se as diferenças constitutivas observadas poderão servir como biomarcadores de resistência a este patogénio, analisou-se o conteúdo em FA de várias espécies resistentes/tolerantes de videira (*V. Riparia*, *V. Labrusca*, *V. Rupestris*, *V. Rotundifolia*, *V. Candicans*, *V. Sylvesteris* e *V. vinifera* cv. Regent) e cultivares suscetíveis de *Vitis vinifera* (*V. vinifera* cv. Pinot noir, Trincadeira e Cabernet Sauvignon). Os nossos resultados apontam para a existência de um padrão discriminatório entre as videiras resistentes/tolerantes e suscetíveis, ao *P. viticola*, sobretudo ao nível dos FA insaturados, nos quais se observa uma menor quantidade de ácido oleico e linoleico (C18:1 e C18:2) e uma maior quantidade em C18:3 nas espécies de videira resistentes/tolerantes.

Palavras chave:
Vitis vinifera, *Plasmopara viticola*, fosfolipase A, sinalização lipídica, ácido jasmónico

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Abbreviations

ABA	Abscisic acid
AVR	Strain-specific avirulent
AzA	Azelaic acid
BABA	β-aminobutyric acid
cDNA	Complementary DNA
cv.	Cultivar
C16:0	Palmitic acid
C16:1*t*	Trans-hexadecanoic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	α-linolenic acid
DAD PLA$_1$	DEFECTIVE IN ANTHER DEHISCENCE PLA$_1$
DBI	Double bond index
DGDG	Di–galactosyldiacylglycerols
ETI	Effector-triggered immunity
FA	Fatty acids
FAD	Fatty acids desaturases
GC	Gas chromatography
hpi	Hours post-inoculation
HR	Hypersensitive response
JA	Jasmonic acid
JA-Ile	JA with isoleucine
JAR1	Jasmonate:amino acid synthetase
LOX	Lipoxygenase
MDA	Malondialdehyde
MeJA	Methyl jasmonic acid
MGDG	Mono–galactosyldiacylglycerols
Mw	Molecular weight
NL	Neutral lipids
OPR3	Oxophytodienoate reductase 3
PA	Phosphatidic acid
PAMP	Pathogen-associated molecular patterns
PA-preferring PLA$_1$	Phospatidic acid-preferring PLA$_1$
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PG	Phosphatidilglycerol
PI	Phosphatidylinositol
pI	Isoelectric point
PIs	Phosphoinositides
pPLA	Patatin-like phospholipase A
PR	Pathogenesis-related genes
PTI	PAMP-triggered immunity
PUFAs	Polyunsaturated fatty acids
qPCR	quantitative real-time PCR

ROS	Reactive oxygen species
SACPD	Stearoyl-ACP desaturases
SA	Salicylic acid
SAR	Systemic acquired resistance
$sPLA_2$	Secretory phospholipase A2
SSI2	Suppressor of salicylic acid insensitivity
TAG	Triacylglycerol
TLC	Thin Layer Chromatography

1. Introduction

Plants are daily exposed to various stress factors, from environmental stressors (eg. temperature, drought) to pathogens attacks. Thus, they have evolved their own defence mechanisms capable of recognizing and respond to stressful conditions. In plant-pathogen interactions, plants recognize either pathogen-associated molecular patterns (PAMPs), that trigger an immune response, called PAMP-triggered immunity (PTI), or strain-specific avirulent (AVR) proteins that interact with plant resistance genes (R) and initiate effector-triggered immunity (ETI). Effector-triggered immunity involves an hypersensitive responses (HR) characterized by programmed cell death, production of reactive oxygen species (ROS) that lead to an oxidative burst and high expression of pathogenesis-related genes (PR)[1–4]. Beside the local resistance, HR, the systemic acquired resistance (SAR) is induced upon pathogen recognition, leading to the production of several signals that after a translocation to distal tissues provide a broad-spectrum resistance[5].

1.1. Fatty acids in plant-pathogen interactions

Fatty acids (FA) are the main component of cells membranes and an important source of energy. Besides their structural and metabolic roles, FA and FA metabolites participate in plant defence, modulating a myriad of signal transduction pathways. In response to stressful conditions FA can influence cell signalling by changing both membrane fluidity and membrane lipid composition.

The major constituent of membrane lipids are the polyunsaturated FA (PUFAs) linoleic and linolenic acids that under pathogen attack can be released by phospholipases and act directly in plant defence as free FA or indirectly, providing substrate for oxylipins production[4,6]. One of the PUFAs-derived products is the monocarboxylic acid 9-oxononanoic acid (ONA) that is generated by the oxidative cleavage of C18 unsaturated FA, present in galactolipids (plastidial membrane lipids), catalysed by ROS. Monocarboxylic acid 9-oxononanoic acid is the intermediate precursor of azelaic acid (AzA), that its turn is an inducer of SAR[7]. High linoleic acid (C18:2) levels were shown to enhance resistance of avocado to *Colletotrichum gloeosporioides attack*[8]. Linolenic acid (C18:3) was also shown to be released from chloroplast lipid membrane and act either directly as signalling molecule by inducing the NADPH oxidase activity and subsequently modulating ROS production and HR[9] or indirectly, by acting in signal mechanisms as substrate for jasmonic acid (JA) biosynthesis[10].

Moreover, the saturated FA palmitic (C16:0) and stearic (C18:0) acids play an important role in the biosynthesis of cuticle compounds, cutin and wax[11]. The cuticle is the first line of plant defence, covering the aerial surface as a barrier between plant cells and the environment, limiting the loss of gasses, water, solutes and participating in plant resistance against pathogens[11–14]. Both C18:0 and oleic acid (C18:1) were shown to be involved in the resistance response of *Arabidopsis* against pathogens. Mutation in SSI2 (suppressor of salicylic acid insensitivity), the major SACPD (Stearoyl -ACP desaturases – convert C18:0 in C18:1) isoform leads to high C18:0 and reduced C18:1 levels promoting a constitutive activation of the salicylic acid (SA) pathway and repression of the jasmonic acid (JA) pathway[15].

1.2. Oxilipins and lipid signalling molecules

Membrane lipids, as sphingolipids and phosphatidylinositol (PI), provides substrates for signalling lipids biosynthesis such as free FA, phosphatidic acid (PA), inositol triphosphate (IP_3), oxylipins and others[16]. Phosphatidic acid besides being precursor for the biosynthesis of complex lipids is a signalling molecule, which modulates the activity of proteins involved in membrane trafficking, Ca^{2+} signalling, oxidative burst, phosphatases, phospholipases and kinases[16–19]. Mono and Di – galactosyldiacylglycerols (MGDG and DGDG) are the main lipid compounds of chloroplast membranes. With a high content of C18:3, these lipids are an important source of substrate for oxylipins biosynthesis[10]. Galactolipids, MGDG and DGDG, regulate SAR in distinct pathways. Digalactosyldiacylglycerols contributes in SA and nitric oxide (NO) biosynthesis, regulating SAR and MGDG has effects down-stream of NO, inducing AzA.

Nitric oxide triggers the synthesis of ROS, that are involved in AzA formation, creating a feedback loop[20] (Figure 1).

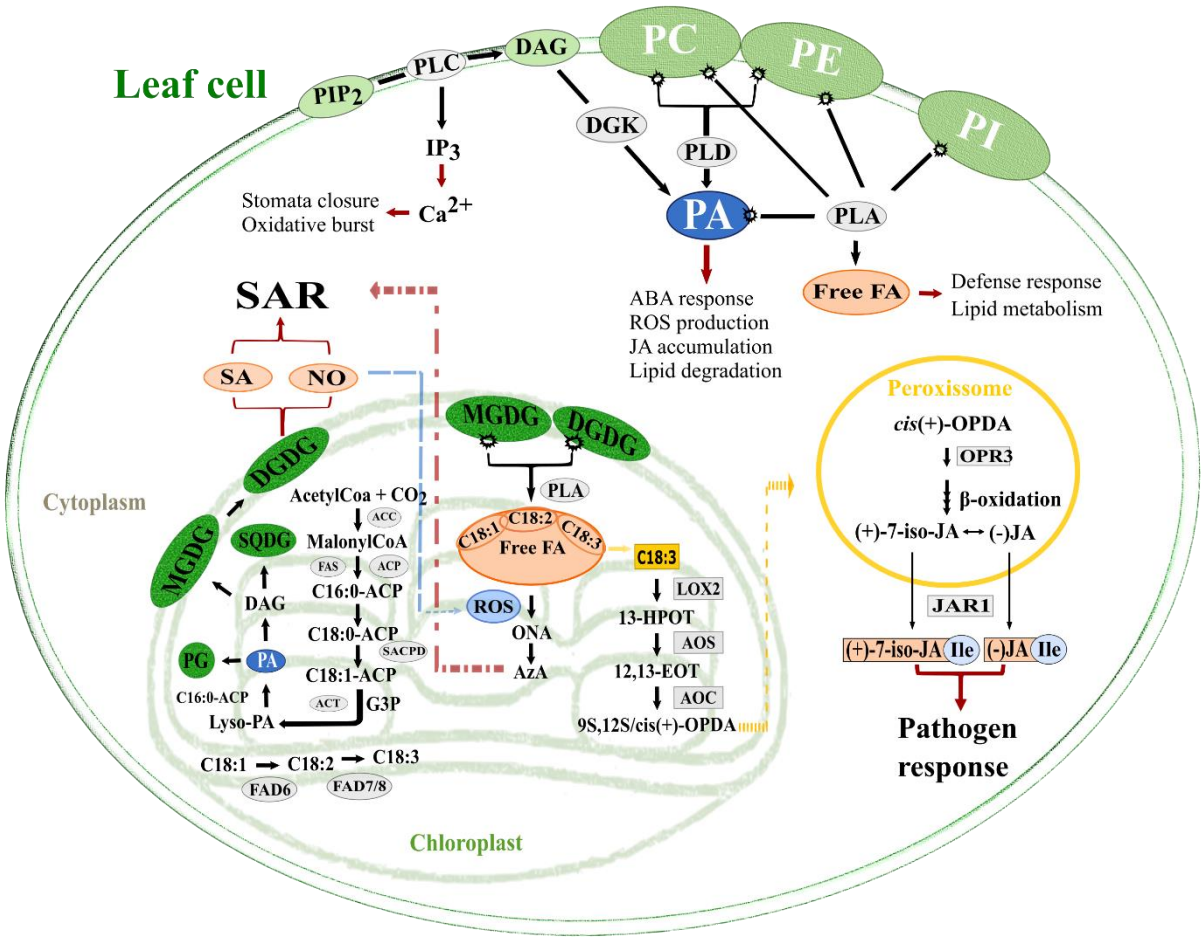

Figure 1 - Scheme of oxilipins and lipid signalling molecules pathways in plant resistance mechanisms against pathogen attack.
Lipid modulation, by phospholipases action, in plant defence response against pathogen attack. FA role in defence mechanisms, upon its release from lipids, serving as signalling molecules or as substrate for oxylipins biosynthesis.
Abbreviations: **9S,13S/cis(+)-OPDA**, (9S,13S)-12-oxo-cis-10,15-phytodienoic acid; **12,13-EOT**, 12,13-epoxy-9-Z,11,15-Z-octadecatrienoic acid; **13-HPOT**, 13S-hydroperoxy-(9Z,11E,15)-octadecatrienoic acid; **(+)-7-iso-JA**, (+)-7-iso-jasmonic acid; **(-)-JA**, (−)-jasmonic acid; **ACC**, acetyl-CoA carboxylase; **ACP**, acyl carrier protein; **ACT**, glycerol-3-phosphate acyltransferase; **ABA**, abscisic acid; **AOC**, allene oxide cyclase; **AOS**, allene oxide synthase; **AzA**, azelaic acid; **C16:0**, palmitic acid; **C18:0**, stearic acid; **C18:1**, oleic acid; **C18:2,** linoleic acid; **C18:3**, α-linolenic acid;**Ca^{2+}**, calcium ; **DAG**, diacylglycerol ; **DGDG**, Di–galactosyldiacylglycerols; **DGK**, diacylglycerol kinase; **FAD 6/7/8**, Fatty acids desaturases 6/7/8; **FAS**, fatty acid synthase; **G3P**, glycerol-3-phosphate; **IP3**, inositol triphosphate; **JAR1**, jasmonates-amide synthetase; **LOX2**, lipoxygenase 2; **Lyso-PA**, phosphatidic acid; **MGDG**, mono–galactosyldiacylglycerols; **NO**, nitric oxide; **PA**, Phosphatidic acid; **PC**, Phosphatidylcholine; **PE**, Phosphatidylethanolamine; **PG**, Phosphatidilglycerol; **PI**, Phosphatidylinositol; **PLA**, phospholipase A; **PLD**, phospholipase D; **ONA**, monocarboxylic acid 9-oxononanoic acid; **OPR3**, oxophytodienoate reductase 3; **ROS**, reactive oxygen species; **SA**, salicylic acid; **SACPD**, stearoyl-ACP desaturases; **SAR**, systemic acquired resistance.

Oxylipins are potent secondary signal molecules that act in plant defence indirectly, amplifying the initial stimulus received by the plant, and directly as anti-microbial compounds. They are originated from PUFAs that suffered a series of oxidative processes[10,21]. Jasmonic acid, one of the most studied oxylipins, is associated in several physiological, developmental and defence responses. Jasmonic acid biosynthesis starts in the chloroplast, where C18:3 is released from membrane lipids, by the action of phospholipases A[4,10,22–24]. Then, C18:3 is oxidized by lipoxygenases, forming 13S-hydroperoxy (9Z,11E,15Z) octadecatrienoic acid (13-HPOT)[25–28]. Further, under the action of the enzymes allene oxide synthase (AOS) and allene oxide cyclase (AOC) is formed the cis- (+) - 12-oxo-phytodienoic acid

(OPDA)[4,28]. In the peroxisome, OPDA is reduced by oxophytodienoate reductase 3 (OPR3) followed by a series of β-oxidations reactions that will originate JA[4,24]. After JA biosynthesis, this oxylipin is exported to cytosol where it is conjugated with the amino acid isoleucine, by the action of jasmonate:amino acid synthetase (JAR1), resulting in bioactive form of JA conjugated with isoleucine (JA-Ile)[29,30] (Figure 1). Accumulation of JA and its volatile methyl ester, methyl JA (MeJA), is observed in response to pathogens and elicitors and precedes an increase in the expression of effector genes[31–33].

1.3. Fatty acid cleavage in stress responses

Phospholipases are a class of enzymes that catalyse the hydrolysis of acyl esters and phosphate esters in phospholipids. The three major groups of phospholipases, A (PLA), C (PLC) and D (PLD), are classified according the position in which they catalyse the hydrolysis on phospholipids. In contrast to phospholipase C and D that act on the polar head of phospholipids, phospholipase A catalyses the hydrolysis of phospholipids into lysophospholipids and free fatty acids, either at the *sn*-1 (PLA_1) and/or *sn*-2 position (PLA_2) of glycerolipids[34]. Free fatty acids may be oxidized by lipoxygenases (LOX) or an α-dioxygenase resulting in the biosynthesis of oxylipins and JA, which play important roles during plant defence signalling[35]. Moreover, PLA_2 activity has been linked to ROS production[36] as well as efflux of vacuolar protons, which triggers a pH-dependent signal for the biosynthesis of phytoalexins[37].

Plant phospholipase A superfamily, comprehends Phospholipase A_1 (PLA_1), Secretory Phospholipase A_2 ($sPLA_2$) and Patatin-like Phospholipase A (pPLA) that is the closest homologue to the animal calcium-independent PLA_2 ($iPLA_2$). The cytosolic PLA_2 ($cPLA_2$), present in animals, has not been identified in plants.

Phospholipase A_1 comprises the defective in anther dehiscence (DAD) and PA-preferring PLA_1 that sharing a highly conserved catalytic centre (GXSXG)[34,38]. The DAD PLA_1 are divided into three classes (I, II and III), according to sequence homology and the presence of N-terminal stretches[38,39]. They are localized in chloroplast, cytosol and mitochondria, respectively, hydrolyses phosphatidylcholine (PC) and MGDG[34,38,39]. While PA-preferring PLA_1 act on PA and has a Mw around 106 kDa[34,39]. They were shown to be involved in a broad spectrum of biological processes, including JA production[23].

Secretory Phospholipase A_2 are enzymes characterized by a highly conserved Ca^{2+} binding loop (YGKYCGxxxxGC) and an active site composed by histidine (his) residue that forms a catalytic his aspartate (asp) dyad (DACCxxHDxC)[34,40,41]. Secretory Phospholipase A_2 were also associated to plant defence responses against pathogens[39].

Patatin-like Phospholipase A are vacuolar nonspecific lipid acyl hydrolases, with conjugated PLA_1 and PLA_2 activity, present in solanaceous plants. Based on genome sequences analysis, plant pPLA have been divided into three classes (I, II and III). These phospholipases are characterized by a catalytic centre (GXSXG), conserved in pPLA and animal $cPLA_2$ and the phosphate-or anion-binding motif DGGGXRG that differs from SGGGXR in $cPLA_2$. The members of pPLA family are associated with the endoplasmic reticulum and the plasma membrane or localized to the cytoplasm except for AtpPLA-I (At1g61850), which showed dual localization to the cytosol and chloroplasts[39,42–44]. Patatin-like Phospholipase A have also been described as involved in responses to both biotic and abiotic stresses[45–47].

1.4. Lipid associated signalling in grapevine downy mildew

Grapevine is currently one of the cultivated crops with higher socioeconomic impact in the world. From over 70 identified *Vitis* species, the domesticated *Vitis vinifera* is the only used for wine production generating revenue over 29 billion in 2016. Portuguese wine industry has a crucial importance in the countries' economy, being Portugal the 11th wine producer worldwide in 2016[48]. The domesticated *Vitis vinifera* used for wine and grape production is highly susceptible to pathogens namely to *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, the downy mildew causing agent. This pathogen was introduced

in Europe, on late 19th century, together with grafts from American *Vitis* that were used to replant vineyards affected by phylloxera[49]. *Plasmopara viticola* is an obligate biotrophic oomycete pathogen that under favourable climate conditions infects grapevine. *Plasmopara viticola* zoospores are released from sporangia and penetrate the leaf through the stomata, forming a substomatal vesicle. In the intercellular spaces of mesophyll tissue, this vesicle forms the primary hypha that differentiates in haustoria, a specialized structure that allow nutrient uptake, plant defence suppression and redirection of host metabolism (Figure 2). Subsequently, when the pathogen completes its life cycle (compatible interaction) the adaxial leaf surface exhibits the characteristics oil-spots[50–52].

Downy mildew causes the most severe epidemics on grapevine crops, with devastating effects. The main disease control strategy is the preventive application of phytochemicals (every 10-15 days on a growing season) leading not only to high environmental and economic problems, but also to the development of fungicide resistant strains of *P. viticola*[53]. Although, all of *Vitis vinifera* cultivars commonly used in viticulture are susceptible to *P. viticola*, several American and Asian *Vitis* species present high tolerance/resistance to this pathogen and are currently being used as genetic sources of resistance in breeding programs[49,54]. The resistant *Vitis* inhibits or limits the infection while in the tolerant interaction the pathogen initiates the infection, but the host are capable of overcome it[55]. Thus, understanding the molecular mechanisms that allow grapevine to establish a successful resistance mechanism is crucial for developing alternative strategies of disease control that allow a sustainable viticulture.

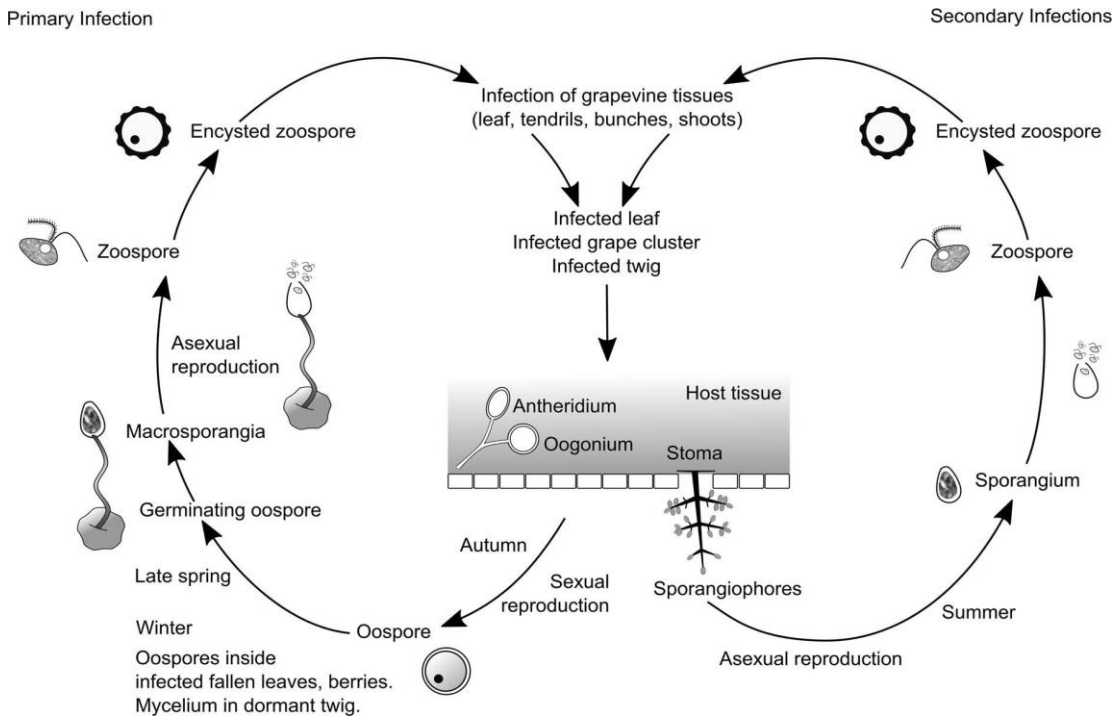

Figure 2 – *Plasmopara viticola* life cycle, adapted from Buonassisi et al., 2017
The *P. viticola* life cycle comprises sexual and asexual reproduction during winter and during the period of grapevine vegetative growth, respectively. In winter, oospores, resulting from the fertilized oogonia by antheridia, are onto fallen leaves and host tissues, infected during the previous season. In spring oospores germinate and form a macrosporangium, where zoospores are released and dispersed onto leaves by wind or rain. In leaf, zoospores swim towards stoma and forms a germinative tube, penetrating the stomata cavity and culminating in an intercellular mycelium. During summer, in stoma occurs sporulation where the sporangia are released and dispersed to the host tissues, getting ready new infection.

The importance of lipids in signalling processes, during the interaction between grapevine and *P. viticola*, has been highlighted in recent works of our group. In the last decade, there was an increasing evidence that jasmonic acid may participate in the grapevine resistance against biotrophs pathogens, such as powdery and downy mildew[56–59]. The over-expression of JA-responsive genes and LOX upon β-aminobutyric acid (BABA) application[56], increase in JA and MeJA levels and over-expression of *AOS* and *AOC* 48 hpi with *P. viticola*[60] and an increase in C18:3 content in inoculated grapevines with *P. viticola*[61] has been shown.

Our group has presented evidences corroborating JA role in defence against *Plasmopara viticola*. In a resistant grapevine genotype (incompatible interaction) there is an early up-regulation of enzymes involved in JA biosynthesis (*lipoxygenase* (*LOXO*), *AOC*, *AOS* and *OPR3*) and a later up-regulation for JA signalling enzymes (*JAR1*) and *coronatine insensitive 1* (*COI1*)), after *P. viticola* inoculation. Moreover, we have shown a higher accumulation of both JA, JA-ILE and SA in the first hours of interaction[62] as well as an up-regulation of genes involved in JA-signalling, SA-signalling and genes involved in crosstalk between SA and JA signalling pathways[62]. Our results provided strong evidences that JA plays an important role in the establishment of the incompatible interaction acting synergistically with SA. Our group has recently shown that both H_2O_2 accumulation and lipid peroxidation occur in the resistant genotype as soon as 6 hours post-inoculation (hpi) with the pathogen[63]. ROS hav been pointed as an important response mechanism in plant resistance against pathogens by strengthening host cell walls via cross-liking glycoproteins and lipid peroxidation[64].

Lipid peroxidation also plays a key role in early defence signalling; by participate in necrosis, cell apoptosis, programmed cell death and JA production[6]. Malonaldehyde and 4-hydroxynonenal (MDA and 4-HNE) are lipid peroxidation products and have also been described as signalling molecules that act under stress conditions[65]. Our group has also shown that major lipid complex-like protein 423 (MLP-like protein 423) were more accumulated in the resistant grapevine genotype[63], these proteins are related to hydrophobic compounds, such flavonoids and FA binding and translocation. Thus, they might be involved in transport lipid and long-distance lipid transport and signalling[66]. Also, plastidial lipid associated proteins also termed as fibrillin, were highly accumulated in the resistant grapevine genotype as soon as 12 hpi[63]. These proteins as act as support for building lipid droplets that contain free fatty acids, carotenoids, phytols, quinones, and other lipophilic compounds and some of them could be damaged due photooxidative conditions. In stressed plants, fibrillins levels were associated to JA production, by storing JA precursors, as C18:3, to produces rapidly JA after oxidative stress[67].

Overall, our previous results highlighted the importance of lipid associated signalling events in the establishment of an incompatible interaction between grapevine and *P. viticola*.

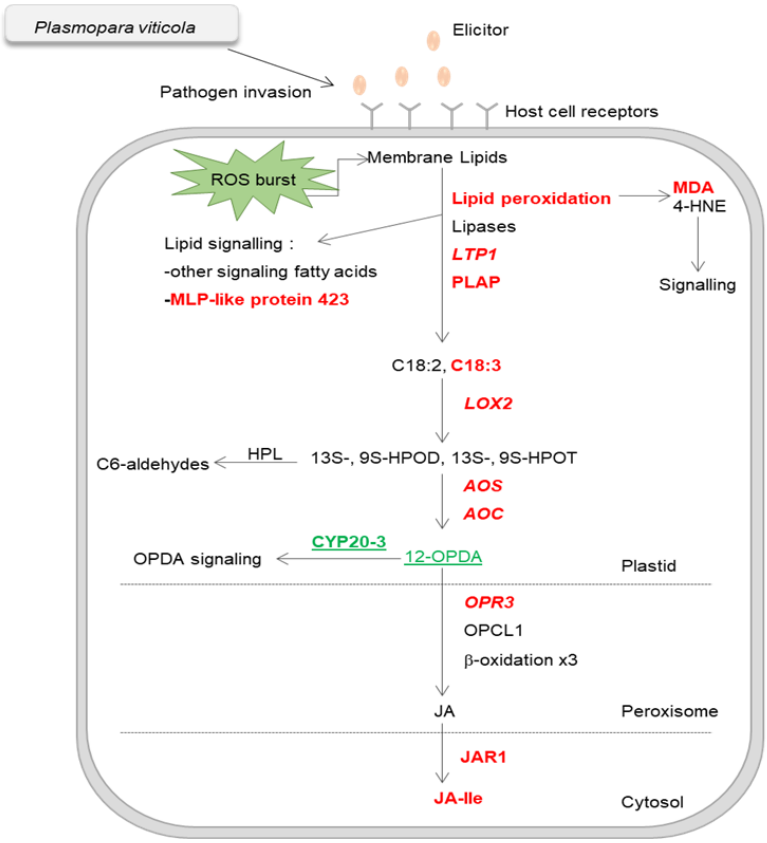

Figure 3 - Overview of the pathways involved in generation of fatty acid-derived signals. Up-regulated genes, accumulated proteins and metabolites in *V. vinifera* cv. Regent are represented in red, down-accumulated proteins are represented in green.

Abbreviations: **C18:1**, oleic acid; **C18:2**, linoleic acid; **C18:3**, linolenic acid; **MDA**, malonaldehyde; **4-HNE**, 4-hydroxynonenal; **LTP1**- lipid transfer protein 1; **PLAP**, plastid lipid associated protein; **9S–HPODE** and **13S-HPODE**, 9S–hydroperoxylinoleic and 13S-hydroperoxylinoleic acid; **9S-HPOTE** or **13S-HPOTE**, 9Shydroperoxylinolenic or 13S-hydroperoxylinolenic acid; **HPL**, hydroperoxide lyase; **AOS**, allene oxide synthase; **AOC**, allene oxide cyclase; **12-OPDA**, 12-oxophytodienoic acid**; OPCL1**, OPC-8:0 CoA Ligase1; **JAR1**, jasmonate resistant 1; **CYP20-3**, peptidyl-prolyl cis-trans isomerase; **MLP-like protein 423**, major latex like protein 423. Adapted from Figueiredo et al., 2017.

1.5. Aims

In our previous works we have shown that JA plays an important role in the establishment of the incompatible interaction between grapevine and *P. viticola*. Up to our knowledge, no studies have been conducted on lipid and FA modulation in the first hours of interactions. In the present study we aimed at monitoring the lipid and fatty acids modulation to comprehend the initial steps of lipid signalling during interaction between grapevine and *Plasmopara viticola*. We have used thin layer chromatography (TLC) and gas chromatography (GC) to characterize FA modulation at the first hours of interaction with *Plasmopara viticola*. We characterized for the first time the phospholipase A superfamily, in *V. vinifera* based on phylogenetical analyses, chromosomal location, subcellular location, gene and protein structure. We have also selected several PLA genes based on the bibliography and proximity to the *P. viticola* resistance associated loci (RPV) to accessed their expression levels in the resistant grapevine genotype, Regent, at three time-points after inoculation with *P. viticola* (6, 12 and 24 hpi).

2. Materials and Methods

2.1. Inoculation Experiments

Plasmopara viticola inoculations were made in greenhouse grown *Vitis vinifera* cv. Trincadeira and Regent plants, as previously described[23]. Briefly, *P. viticola* sporangia were collected from symptomatic leaves from greenhouse infected plants after an overnight incubation in a moist chamber at room temperature. Sporangia were carefully collected by brushing, dried and stored at −25 °C. Their vitality was checked by microscopy[29]. A suspension containing 10^4 sporangia ml^{-1} was used to spray the abaxial leaf surface while controls were made by spraying the leaves with water (mock inoculations). After inoculation, plants were kept for 8 h in a moist chamber (100% humidity) and then kept under greenhouse conditions during the inoculation time course. The third to fifth fully expanded leaves below the shoot apex were collected at 6, 12, and 24 hpi, immediately frozen in liquid nitrogen and stored at −80°C. Three independent biological replicates were collected for each condition (inoculated and mock inoculated).

2.2. Lipid analysis

Ground leaves were boiled in water for 5 min to inactivate lipolytic enzymes. The extraction of lipophilic compounds was performed using a mixture of chloroform/methanol/water (1:1:1, v/v/v), as previously described[68]. Lipid classes' separation was carried out at 6 hpi by thin layer chromatography (TLC) on silica plates (G-60, Merck, VWR), as previously described[69,70] using tow solvents system, chloroform/methanol/acetone/acetic acid/water (100/20/40/20/8, v/v/v/v/v) that separates the different polar lipids[71], the neutral lipids separation from polar lipids was carry out by the petroleum ether/ethyl ether/acetic acid (70/30/0.4, v/v/v) solvent system[68]. Lipids bands were visualized with primuline (0.01% in 80% acetone) under UV light, and scraped off. Fatty acids methyl esters (FAME) were prepared by trans-methylation of fatty acids with methanol:sulfuric acid (97.5:2.5, v/v). Fatty acids quantitative analysis was performed using gas chromatography (GC) (430 Gas Chromatograph, Varian) at 210ºC, equipped with hydrogen flame ionization detector, heptadecanoic acid (C17:0) as an internal standard. The double bond index (DBI) was calculated as in Equation 1.

$$DBI = \frac{(\%\text{monodienoic acids}) + 2(\%\text{dienoic acids}) + 3(\%\text{trienoic acids})}{100}$$

Equation 1 - Calculation of double bound index

2.3. Identification and retrieval of grapevine PLA sequences

Phospholipase A genes and amino acid sequence identification was performed using *Arabidopsis thaliana* and *Oryza sativa* (rice) PLA proteins sequences as a query for blast searches at NCBI BLAST tool[72] (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *Arabidopsis* and rice PLA members were searched and the sequences were retrieved from TAIR[73] (https://arabidopsis.org) and RGAP (https://rice.plantbiology.msu.edu) databases, respectively. To find additional *Vitis vinifera* PLA, a search restricted to "*Vitis*" was performed on NCBI (https://www.ncbi.nlm.nih.gov/), using the PLA conserved motifs (GxSxG, RYGKYCGxxxxGC, LDACCxxHDxCV, DGGGxRG, GTSTG, SAAPTY, DGGxxANN) as query. The putative grapevine PLA sequences were confirmed on NCBI and CRIBI (http://genomes.cribi.unipd.it) (November 2017).

2.4. Domain structure analysis, sequence properties, subcellular location prediction and chromosomal location

Domain and clan determination was performed using Pfam (http://pfam.xfam.org/). Molecular weight (Mw) and isoelectric point (pI) were predicted using the ProtParam tool from ExPASy[74] (http://web.expasy.org/protparam/). Subcellular location of proteins was predicted using TargetP[75]

(http://www.cbs.dtu.dk/services/TargetP/), Localizer[76] (http://localizer.csiro.au/), Predotar[77] (https://urgi.versailles.inra.fr/predotar/) and Blast2GO version 3.3[78] (https://www.blast2go.com/), The Map Viewer tool from NCBI (http://www.ncbi.nlm.nih.gov/mapview/) was used to map PLA genes in *V. vinifera* chromosomes. Blast2GO tool was used to predict the Putative function. The physical map constructed with grapevine phospholipases gene location was also compared to a genetic linkage map representing *Plasmopara viticola* resistance (RPV) QTLs in grapevine[71–79], in order to access the location of grapevine phospholipases within these loci. All the molecular predictions were manually curated and compiled.

2.5. Phylogenetic analysis

The alignment of *V. vinifera* and *Arabidopsis* PLA protein sequences was made with MAFFT software, with the L-INS-I option version 7[88] (http://mafft.cbrc.jp/alignment/software/). The sequences editions were performed with Jalview software (http://www.jalview.org/). A maximum likelihood (ML) phylogenetic analysis was performed with RAxML-HPC v.8, on CIPRES Science Gateway[89] (https://www. phylo.org), with the following parameters: protein substitution model PROTCAT; protein substitution model + BLOSUM62; bootstrap 1000 iterations with rapid boot strap analysis (−fa). Both trees were viewed on FIGTree (http://tree.bio.ed.ac.uk/software/figtree/) and edited on Inkscape (http://www.inkscape.org/).

2.6. RNA extraction and cDNA synthesis

Total RNA was isolated from *V. vinifera* cv. Regent inoculated and mock inoculated samples using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) and the residual genomic DNA was hydrolyzed with the On-Column DNase I Digestion Set (Sigma-Aldrich, USA) as indicated by the manufacturer. RNA concentration and purity were determined at 260/280 nm using a NanoDrop-1000 spectrophotometer (Thermo Scientific), while its integrity was analyzed by agarose gel electrophoresis. Prior to complementary DNA (cDNA) synthesis, all samples were analysed for genomic DNA contamination by a quantitative real time Polymerase Chain Reaction (qPCR) of a reference gene on crude RNA[90] . Complementary DNA was synthesized from 2.5 µg of total RNA using RevertAid®H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo$(dT)_{23}$ primer (Fermentas, Ontario, Canada), following the manufacturer's instructions.

2.7. Quantitative real time PCR

qPCR experiments were performed using the Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, Canada) following manufacturer's instructions. Reactions were performed in the StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). A final concentration of 2.5 mM $MgCl_2$ and 2 µM of each primer were used in 25 µL volume reactions, with 4 µL of cDNA as template. Each set of reactions included a control without cDNA template. Primer sequences and amplification details are provided in Table 1. For all genes, thermal cycling started with a 95 °C denaturation step for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at gene specific temperature (Table 1) for 30 seconds. Dissociation curve analysis was performed to confirm single product amplification and the existence of non-specific PCR products (Supplementary data 1). Three biological replicates and two technical replicates were used for each sample. Gene expression (fold change) was calculated as described in[91] . *Elongation Factor 1-alpha* (*EF1α*), *Ubiquitin-conjugating enzyme* (*UBQ*) were used for expression data normalization as previously described[92].

2.8. Statistical analysis

Due to the lack of data normality and homogeneity of variances, the statistical analysis of the data was based on non-parametric tests. In order to compare FA, lipid profile and qPCR data in inoculated and non-inoculated samples, Statistical analysis was performed by the Mann-Whitney U test using IBM® SPSS® Statistics software (version 23.0; SPSS Inc., USA). Results yielding $p < 0.05$ were considered statistically significant

The differences among susceptible and resistant group (section 3.9) groups were evaluated using Similarity Percentage (SIMPER) test and Principal Component Analysis (PCA) with Primer 6 software[93]. Samples were grouped according to their degree of similarity as provided by SIMPER. This test is frequently used to compare different communities using the intrinsic provided characteristics of each community, being insensitive to heterogeneous data[94–96]. The application of the SIMPER analysis was used to identify which fatty acid variables were responsible for the separation of the different groups.

Table 1- Reference and target genes transcripts primer sequences, amplicon length and qPCR analysis.
EF1α and *UBQ* were used as reference genes[97]. *VvipPLA-IIα1/2*, *VvipPLA-IIδ1* and *VvipPLA-IIδ3* were not detected in inoculated samples.
* No discrimination between these genes sub-group.

Gene name		Abbreviation	NCBI Accession Number	Primer Sequence	Amplicon length (bp)	Ta (°C)	Tm (°C)	Amplification efficiency (E)
Elongation Factor 1-alpha		*EF1α*	XM_002284888.2	Fw: GAACTGGGTGCTTGATAGGC Rev: ACCAAAATATCCGGAGTAAAAGA	149	60	79.45	1.89
Ubiquitin-conjugating enzyme		*UBQ*	XM_002284161.3	Fw: GAGGGTCGTCAGGATTTGGA Rev: GCCCTGCACTTACCATCTTTAAG	75	60	78.65	1.97
	Group I	*VviPLA$_I$-Iβ1*	XM_002270956.4	Fw: TCCCTAGTGCTCATTCCGAT Rev: TTGAAGAAGACTGGCGACCT	122	58	78.81	2.05
		VviPLA$_I$-Iγ1	XM_002281871.3	Fw: ACTGCGGGACCTGTAGATTT Rev: TTAGGCCATCGGGACTTCTT	142	60	80.53	2.00
	Group II	*VviPLA$_I$-IIδ*	XM_002281059.4	Fw: ATGAGGAGGGGAGTAGCAG Rev: TCTGGGGTCACCTGAAGTG	104	60	77.17	1.94
Secretory Phospholipase A2		*VvisPLA$_2$*	XM_002282786.3	FW: CAGGTGCGCCAATAGGACA Rev: CGGCGACTGCGAAGAAGAA	89	60	81.71	1.91
Patatin-like phospholipase A	**Group I**	*VvipPLA-I* *	XM_010657059.2	FW: TTGTGCCTGAACCCCGTGT Rev: CCTTTCTTCCCAACCTGTCT	109	60	80.48	2.06
	Group II	*VvipPLA-IIα1/2* *	XM_002282445.3	FW: TTCATACCTCCATCGCTCC Rev: CCTCTTATTCCACCTCCAT	Not detected due to low transcript			
		VvipPLA- IIβ *	XM_019216314.1	FW: CTGCAGCACCAACATACTTT Rev: CTGCGACACCACCATCAATA	96	58	75.59	1.90
		VvipPLA-IIδ1	XM_002281762.4	FW: ATAACAACGGGTCCACTCCA Rev: ATCCGAGGGCCTGAAATACA	Not detected due to low transcript			
		VvipPLA-IIδ2	XM_002277282.3	FW: GACTCCGTGAGTCAATGGC Rev: TGGGGTGAAGAAGTGGTCAA	116	60	75.57	2.02
		VvipPLA-IIδ3	XM_002277269.2	FW: CGGAATCAAGCCTTTTTACC Rev: TCATATTTTGGCCCTGTTAG	Not detected due to low transcript			
	Group III	*VvipPLA-IIIβ*	XM_010660108.2 XM_019224348.1	FW: ACGTTGAAGCCGGTGTTGAT Rev: GGAAGTCGAAGCTGTCTGTT	100	60	82.6	1.92

3. Results and Discussion

3.1. Analysis of total of fatty acids composition in *V. vinifera* cv. Trincadeira and cv. Regent at 6, 12 and 24 hpi

Free FA and their derivates may act as modulators of a multitude of signal pathways in plant defence[6]. Polyunsaturated FA are the major constituent of lipid membranes and are released under the activity of lipases upon pathogen attack[6]. They act in defence directly as free FA or indirectly providing substrate for oxylipin production[4,6,98]. In our previous studies we have shown that grapevine interaction with the biotrophic pathogen *P. viticola*, leads to an up-regulation of JA biosynthesis and JA-Ile accumulation, H_2O_2 accumulation and lipid peroxidation in the establishment of the incompatible interaction (resistance)[28,62,63]. However, no studies were yet conducted in membrane lipid modulation after *P. viticola* inoculation, thus we have evaluated FA modulation in two *V. vinifera* cv. leaves, Regent (resistant to *P. viticola*) and Trincadeira (susceptible to *P. viticola*), at 6, 12 and 24 hpi.

In the course of plant development, C18:0 is synthesized from C16:0. A progressive desaturation of C18:0 leads to the formation of C18:1, C18:2 and C18:3 [4]. Both C16:0 and C18:0 levels suffer alterations in the resistant genotype Regent after inoculation, whereas no alteration was observed in the susceptible genotype Trincadeira. In Regent, C16:0 content decreased at 6 and 12 hpi, while C18:0 levels decreased at 6 hpi (Figure 4A, B), followed by a change in unsaturated FA content.

In Regent at 6 hpi the content in the unsaturated FAs C18:1 and C18:2 increased. C18:1 was described to participate in plant defence, by stimulation or binding to proteins that promote anti-cell-death[99], regulates NO synhtesis[100] or AZA biosynthesis contributing to systemic acquired resistance (SAR)[101,102]. Moreover, the increase of C18:2 at 6 hpi may induce a steric obstruction, leading to a decrease in water and solutes changes between cells and environment which may in turn lead to pathogen nutrients privation[103], promoting the establishment the incompatible interaction.

At 12 and 24 hpi both C18:1 and C18:2 content decreased and the C18:3 increased (Figure 4B, C). These results suggest that a conversion of C16:0 in C18:0 may be occurring, followed by desaturation of C18:0 leading to the accumulation of C18:3. This is in accordance to the previously reported by Ali and co-workers[104], that detected a C18:3 accumulation in Regent inoculated with *P. viticola*. In the later time-point it is also visible an increment of the ratio C18:3/C18:2, which suggests that a desaturation process of unsaturated fatty acids C18 still occurs at 24 hpi (Figure 4C, E). High levels of unsaturated lipids had previously been associated to resistance against fungal and bacterial pathogens[3,5,4,7,6]. For example, it was observed by Wang, C. *et al* that an accumulation of C16:1 in transgenic tomato and eggplants is associated to increased resistance to powdery mildew[2].

Membrane fluidity is also affected by lipid unsaturation degree. Membranes with a higher content of unsaturated fatty acids are more fluid than membranes with a lower unsaturated lipids content[105]. When the ratio between saturated and unsaturated FA increases, changes in membrane fluidity occur, leading to an alteration in permeability[103]. Another parameter that reflects membrane fluidity is the double bound index (DBI). When DBI increases, membrane fluidity also increases.

Prior to pathogen challenge, when comparing the content in saturated and unsaturated lipids in the two grapevine genotypes, we observed that the unsaturated/saturated FA ratio is higher in Trincadeira than in Regent, together with a high DBI (Figure 4F). Our results suggest the resistant genotype presents a more rigid membrane, when compared to the susceptible genotype, which may physically hinder pathogen entrance

After inoculation, no alterations were seen on both ratios in Trincadeira. However, in Regent at 6 and 12 hpi, both DBI and unsaturated/saturated ratios increase, leading us to hypothesize that after pathogen

challenge, there is an increase on membrane fluidity that may be important to avoid membrane damage after lipid hydrolysis as a response to the pathogen.

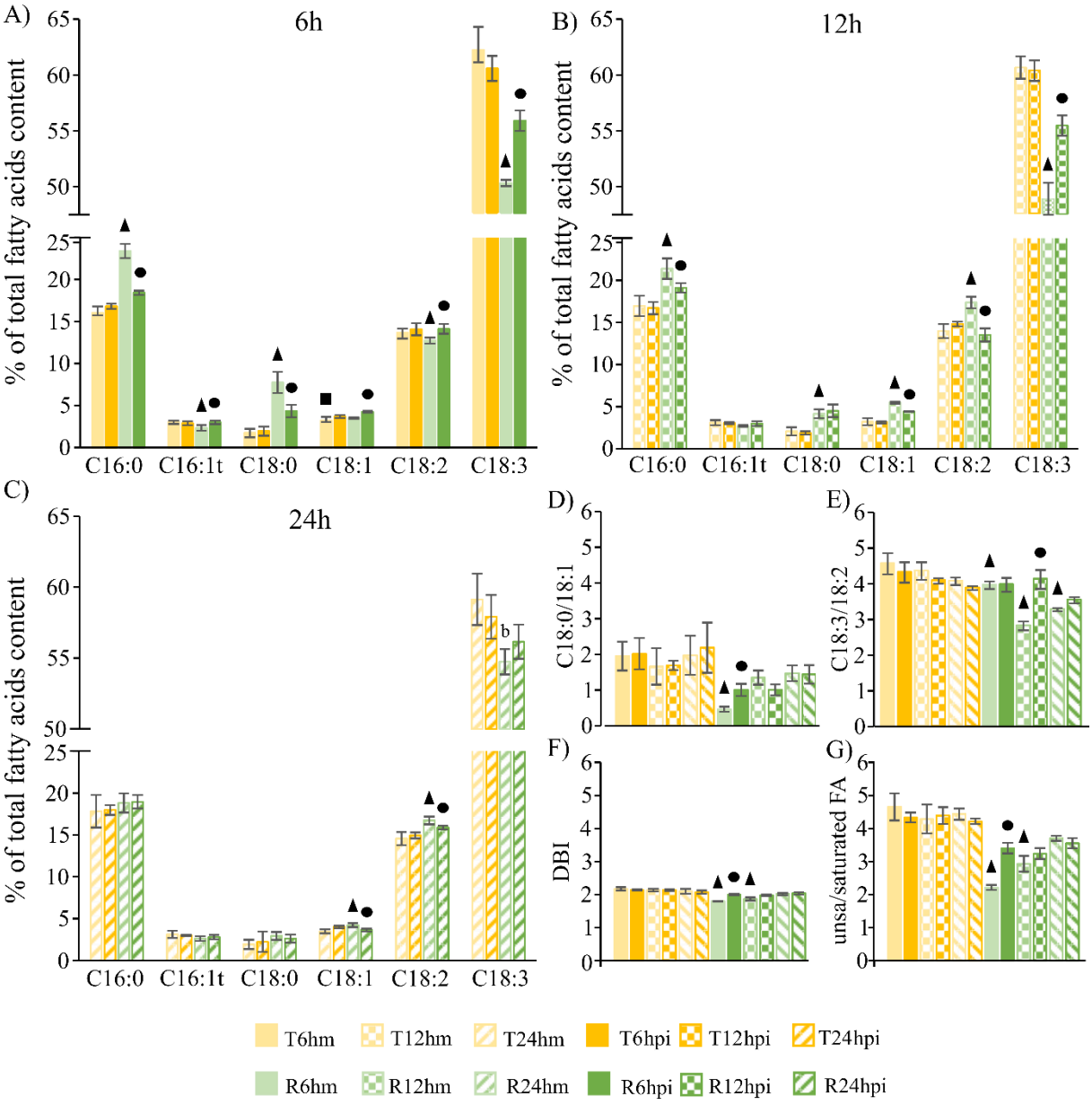

Figure 4 - Gas Chromatography measurements of total fatty acid percentage between *V. vinifera* cv. Trincadeira and Regent at 6, 12 and 24 hpi.
Total of FA content between Trincadeira and Regent at: A) 6 hpi, B) 12 hpi, B) 24 hpi with *P. viticola*; D) ratio C18:0/C18:1, E) ratio C18:3/C18:2, F) DBI and G) ratio of unsaturated/saturated FA between Trincadeira and Regent and the three time-points. Symbols marks represents the statistically differences between (■) (Tm6h vs Ti6h, Tm12h vs Ti12h, Tm24h vs Ti24h), (▲) (Tm6h vs Rm6h, Tm12h vs Rm12h, Tm24h vs Rm24h) and (●) (Rm6h vs Ri6h, Rm12h vs Rmi2h, Rm24h vs Ri24h) at $p<0.05$. Values correspond to average relative percentage ± standard error, n = 4.

3.2. Lipid modulation during the first hours of interaction with *P. viticola*

The major alterations on fatty acid profile occur at 6 hpi with *P. viticola*. We have further selected this time-point to evaluate the changes in lipid classes in both grapevine genotypes. We were able to discriminate several polar lipids, such as PC, phosphatidylethanolamine (PE), MGDG and DGDG, phosphatidylglycerol (PG), PA, PI, neutral lipids such as triacylglycerol (TAG) and free FA (Figure 5). The most abundant lipids on grapevine leaves were the plastid lipids, MGDG and DGDG, followed by extra-plastidial lipids, PC and PE. In lower amounts, we have identified the storage lipids, TAG, signalling lipids, PA, PI and free FA.

Prior to pathogen inoculation, both grapevine genotypes are innately different on leaf lipid composition. The resistant genotype, Regent, presents lower content of both MGDG and DGDG and higher content on all other lipid classes (PG, PC, PE, PI, PA, Free FA. TAG and other lipids) when compared to the susceptible genotype, Trincadeira. After *P. viticola* inoculation, both lipid profile and FA content of these two genotypes exhibit distinct patterns of modulation.

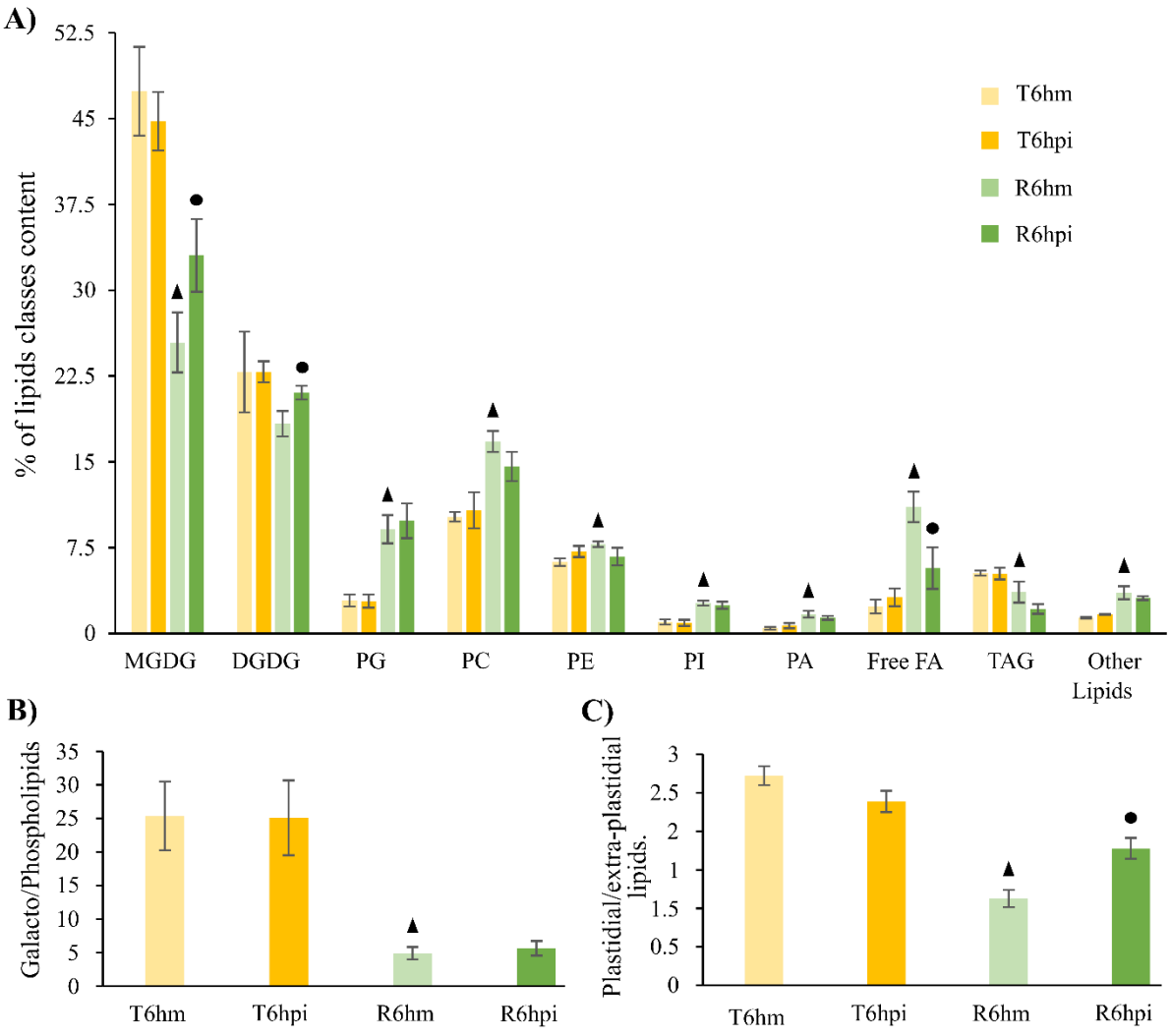

Figure 5- Lipid composition of *V. vinifera* cv. Trincadeira and Regent inoculated leaves with *P. viticola* at 6 hpi. A) Total of lipids content; B) ratio galactolipids/phospholipids; B) plastidial lipids/extra-plastidial lipids. Symbols marks represents the statistically differences between (■) (Tm6h vs Ti6h) – no significant differences were found, (▲) (Tm6h vs Rm6h) and (●) (Rm6h vs Ri6h) at $p<0.05$. Values correspond to average relative percentage ± standard error, n = 3.

Regent presents a higher content in PC and PE, comparatively to Trincadeira (Figure 5A). In Regent leaves, after inoculation, a tendency to decrease in these membrane lipids occurs, while in Trincadeira a tendency to increase in PE content is observed (Figure 5A). The hydrolysis of structural membrane phospholipids, such as PC and PE, by PLD contribute mostly to the PA synthesis[108], thus the decrease of both lipid classes after inoculation may be associated to a further biosynthesis of lipid-related signalling molecules when plant is under stress. Phosphatidic acid is both a glycerolipid metabolic precursor and an important signalling molecule, that regulates developmental, physiological processes and stress responses[109]. The role of PA includes the modulation of a wide range of activities, like phosphatases, phospholipases, kinases, proteins involved in Ca^{2+} signalling, membrane trafficking and oxidative burst[21]. In leaves, a direct application of PA induces PR gene expression and cell death[110,111]. Phosphatidic acid also promotes stoma closure, by binding to the protein phosphatase ABI1, the negative regulator of the ABA response and inhibiting it[112]. This lipid also provides substrate for oxylipins biosynthesis[18]. In the absence of pathogen challenge, PA content in Regent is higher than in Trincadeira, (Figure 5A). After inoculation, its content does not present differences in both cultivars (Figure 5A). The lack of variation could be explained by the fact that the PA biosynthesis may rely on the slower PLD pathway, instead of the PLC and DGK pathways, which only takes minutes[108].

Free FA is originated from membrane glycerophospholipids, when its hydrolysis catalysed by phospholipases occurs. Free FA may act as a second messenger or as precursor of various oxylipins such as JA, with free C18:3 as substrate[39,113]. Also, free FA triggers a wide range of cellular responses, such as modulating H^+-ATPase in plasma membrane, that leads to cell wall acidification and activation of Mitogen Activated Protein Kinases (MAPK) protein kinases[39] or oxidative burst triggering[114].
Constitutively, the resistant grapevine genotype presents a higher free FA content (Figure 5A). Upon pathogen challenge, the content of free FA decreased in Regent, while in Trincadeira no significant alterations were observed (Figure 5A). In resistant genotype, the presence of higher free FA content by itself could be a significant factor to trigger a faster defence response in the moment of the interaction with pathogen. The decrease on free FA decrease after inoculation may suggest that these FA are involved in plant defence mechanisms, mainly as substrates for signalling molecules such as JA.

Regent leaves present a lower content in galactolipids, MGDG and DGDG, comparatively to Trincadeira (Figure 5A). After inoculation, there were no significant variations in Trincadeira, unlike the resistant genotype, where is observed an increase in galactolipids content as well as in C18:3 (Figure 5A; Figure 6A, B). Moreover, the content in the FA C18:0, C18:1 and C18:2 decreased in MGDG and DGDG, while the content in C16:0 decreased only in MGDG (Figure 6A, B). This FA modulation demonstrates that a desaturation of C18:0 may be occurring, leading to the accumulation of C18:3 in both galactolipids (Figure 6A, B). These galactolipids have been described in the establishment of the defence responses by regulating SAR in distinct pathways. DGDG contribute to SA and NO biosynthesis, whereas, MGDG was described to contribute to AzA biosynthesis[20].

PG, a thylakoid lipid, has an important role in photosynthesis and is characterized by the presence of C16:1t[115]. In *Gracilaria chilensis*, PG can also provide PUFAs for the biosynthesis of oxylipins[116]. In our results, Regent presented a higher constitutive level of PG that was not altered after inoculation (Figure 5A). In the absence of pathogen challenge, PG presented a higher content of C18:1 and lower content of C18:3 when compared to the susceptible genotype. Although lipid content does not vary with pathogen inoculation of Trincadeira leaves, in Regent, FA composition suffers alterations, showing a decrease in C18:0 and an increase of C18:3 (Figure 6C). This decrease in C18:0 is possibly due to a conversion of this FA in PUFAs, resulting in an increase in C18:3.

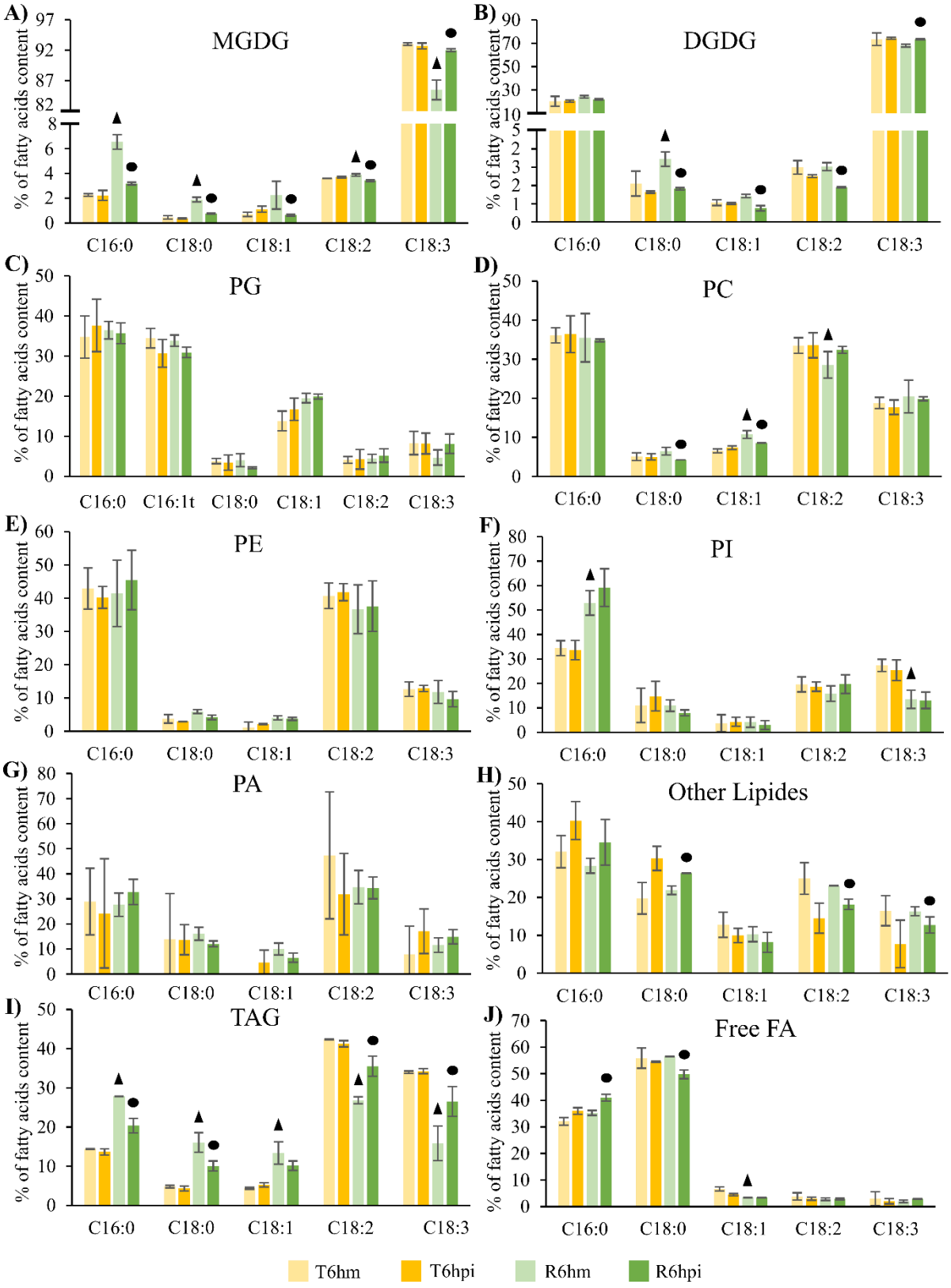

Figure 6 - Fatty acids content in each lipid class of *V. vinifera* cv. Trincadeira and Regent inoculated leaves with *P. viticola* at 6 hpi.

Percentage of total fatty acids present in A) monogalactosyldiacylglycerol (MGDG); B) digalactosyldiacylglycerol (DGDG); B) phosphatidylglycerol (PG); D) phosphatidylcholine (PC); E) phosphatidylethanolamine (PE); F) phosphatidylinositol (PI); G) phosphatidic acid (PA); H) free FA; I) triacylglycerol (TAG); J) other lipids. Symbols marks represents the statistically differences between (■) (Tm6h vs Ti6h, – no significant differences were found, (▲) (Tm6h vs Rm6h) and (●) (Rm6h vs Ri6h) at $p<0.05$. Values correspond to average relative percentage ± standard error, n = 3.

3.3. Identification and characterization of phospholipases A genes in grapevine

Phospholipases A superfamily in plants comprise a group of enzymes involved in several important cellular functions such as cell gravitropism, elongation, anther dehiscence and defence signalling as JA biosynthesis[22,41,41,47,117]. PLA have been previously characterized in some plants species, like *Arabidopsis thaliana* and rice[44,118]. A total of 27 PLA in *Arabidopsis thaliana* and 31 PLA in rice were identified[39,118]. Since grapevine PLA family remains unknown, we performed its characterization, considering the importance of this family in the biosynthesis of oxylipins and jasmonic acid, which play important roles during plant defence signalling[35].

Grapevine PLA genes were identified by homology search using the PLA proteins sequences of *Arabidopsis* and rice, as a query for a BLAST against the *Vitis vinifera* genome and the PLA conserved motifs, as the catalytic centre GXSXG present in PLA_1[34,38], the $sPLA_2$ Ca^{2+} binding loop YGKYCGxxxxGC and the active site DACCxxHDxC[40,41] and the pPLA anion-binding motif DGGGXRG and esterase box GXSXG motif[44]. A total of 41 PLA genes were identified in *V. vinifera* (Supplementary data 2). All the identified genes were mapped and are unevenly distributed in 9 of the 19 *V. vinifera* chromosomes (Figure 7Figure 7). The chromosomes 7, 10 and 18 are the ones where most of the PLA genes are represented. No PLA genes were detected in chromosomes 1, 2, 3, 4, 6, 8, 9, 14, 16, 17 and 19, and the specific location of 2 of the 41 grapevine PLA genes is still unknown (Figure 7Figure 7).

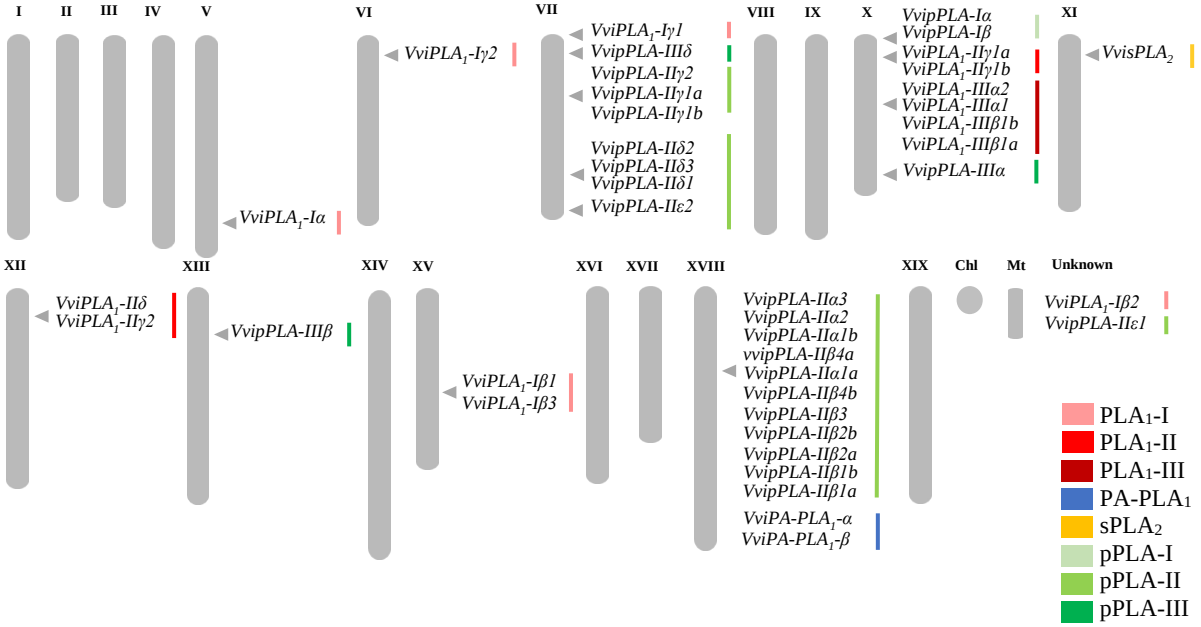

Figure 7 - Locations of *V. vinifera* PLA genes in chromosomes.
Proposed *Vitis* PLA nomenclature is shown in each chromosome.

3.4. Phylogenetic analysis of grapevine phospholipases A

Phylogenetic analysis of the 41 grapevine PLA was performed in order to find relation between PLA proteins (Figure 8). In *Arabidopsis*, the PLA superfamily is composed by 27 members: twelve DAD PLA_1[22], one PA-preferring PLA_1[117], four $sPLA_2$[41,119] and ten pPLA members[120].

To uncover how the grapevine PLA members are divided according to this group nomenclature in *Arabidopsis*, we performed a phylogenetic analysis combining grapevine and *Arabidopsis* PLA (Supplementary figure 3). With this analysis, we also recommend a nomenclature for the grapevine PLA based on sequence similarity with *Arabidopsis* PLA[39,121] (Supplementary data 3).

The analysis leads to the grapevine PLA division in three major classes namely PLA_1, $sPLA_2$ and pPLA (Figure 8; Supplementary data 3). The PLA_1 class comprises DAD-PLA_1 and PA-preferring PLA_1 families. DAD-PLA_1 is divided into three groups, I, II and III, comprising 5, 4 and 4 members, respectively (Figure 8; Supplementary data 3A). PA-preferring PLA_1 is constituted by 2 members (Figure 8; Supplementary data 3A). Secretory PLA_2 is the PLA family less represented with only one member (Figure 8; Supplementary data 3B). On the other hand, pPLA is the most represented family, with 24 members divided into three groups, I, II and III, with 2, 19 and 3 members, respectively (Figure 8; Supplementary data 3C). The PLA members distribution in each group was based on degree of evolutionary relatedness. The phylogenetic relationship between grapevine and *Arabidopsis* PLA superfamily, demonstrated by the degree of relatedness, suggests the existence of common ancestry in these two species, allowing the classification and nomenclature of grapevine PLA (Supplementary data 3).

3.5. Gene structure analysis

The exon-intron structure analysis of grapevine PLA genes reveals a pattern that allow discriminate the members of each PLA group. The number of introns varies between 0 and 20, being 24% of the grapevine PLA genes intronless and around 15% presenting a high number of introns (12 to 20), (Supplementary data 2). All PLA genes without introns belong to the DAD-PLA_1 family, which was also observed in *Arabidopsis*[34,118]. In *Arabidopsis* and rice the PLA members belonging to group I of DAD PLA_1 are intronless[120,122]. In grapevine this intronless pattern is not observed in DAD PLA_1 group I, due to the presence of one intron in VviPLA1-Iγ1 (Supplementary data 2), possibly due to an evolutionary process, but is found in group III (Supplementary data2). Both members of group I of the pPLA family have 18 introns, a value much higher than that observed in *Arabidopsis*[120,122]. On the other hand, the pPLA groups II and III, such as in rice[118], does not present a distinctive intron-exon pattern (Supplementary data 2).

3.6. Protein properties

A prediction of the protein properties of grapevine PLA (Mw, pI, conserved motifs, domains and subcellular location) was performed. Grapevine PLA proteins have a wide range of molecular weight, between 16 and 146 kDa, that varies accordingly to the *Arabidopsis* and rice PLA family[34,118]. The DAD-PLA_1 family proteins have a Mw range between 40 and 60 kDa and the PA-preferring PLA_1 proteins have both around 109 kDa. In pPLA family the Mw is between 40 and 50 kDa with exception for the pPLA group I that the 2 proteins have 118 and 146 kDa. The PLA with the lowest Mw is the secretory PLA with only 16 kDa.

Grapevine PLA have a theoretical pI between 5.02 and 9.51. These pI values are comparable to what is found in *Arabidopsis*, for example DAD1 (AT4G16820) have a pI of 9.737, DAD2 (At4g18550) 5.5, pPLA I (At1g61850) 5.7448, pPLA III (At4g29800) 9.47 and PLA1 (At1g31480) 5.3 (predicted from protein sequence with ProtParam tool from ExPASy[123] (http://web.expasy.org/protparam/).

Each PLA family have highly conserved motifs that are a distinctive feature. We have performed a multiple sequence alignment to identify the consensus and conserved motifs in each grapevine PLA family (Figure 9). All *V. vinifera* PLA_1 share the highly conserved catalytic centre GXSXG[34,38], (Figure 9A, B). The $sPLA_2$ showed the highly conserved Ca^{2+} binding loop YGKYCGxxxxGC and the active site DACCxxHDxC[40,41], (Figure 9C). In patatin-like PLA, the anion-binding motif DGGGXRG was found in all the proteins, whereas the esterase box GXSXG[44] is present only in pPLA groups I and II. In pPLA group III, the serine amino acid residue is replaced by a glycine presenting a non-canonic esterase box GxGxG, instead GxSxG. Specific motifs such as SAAPTY and DGGxxANN[118] are present in all pPLA (Figure 9D). The presence of these conserved motifs are in accordance to the previously described in rice PLA[118].

Concerning *V. vinifera* PLA domains, all DAD-PLA_1 present a lipase 3 domain (Supplementary data 2). The PA-preferring PLA_1 contain the DDHD domain, a conserved metal-binding site often seen in phosphoesterase domains (Supplementary data 2). All pPLA contain the characteristic patatin domain, whilst the group I has an additional domain, the armadillo (ARM) repeats (Supplementary data 2). Until now, no characteristic domain was identified for the $sPLA_2$.

The prediction of the PLA subcellular location, demonstrate that about 60% of the DAD-PLA_1 are located in the chloroplast. The protein members of DAD-PLA_1 group I and group III are predictably located in chloroplast. The DAD-PLA_1–II are divided between chloroplast and mitochondria (Supplementary data 2). The PA-preferring PLA_1 is putatively located in the vacuole membrane (Supplementary data 2). The major of the pPLA are located in cytosol, comprising all group II members, while for group III and for pPLA group I and III was not possible to predict the subcellular location for its members (Supplementary data 2). The subcellular predictions does not totally correspond with the previous PLA characterizations that predicated that the DAD-PLA groups I, II and III are found in chloroplast, cytosol and mitochondria, respectively[22] and the patatin-like PLA are mainly located in cytoplasm, with exception for the pPLA-I that was found in both cytoplasm and chloroplast[34].

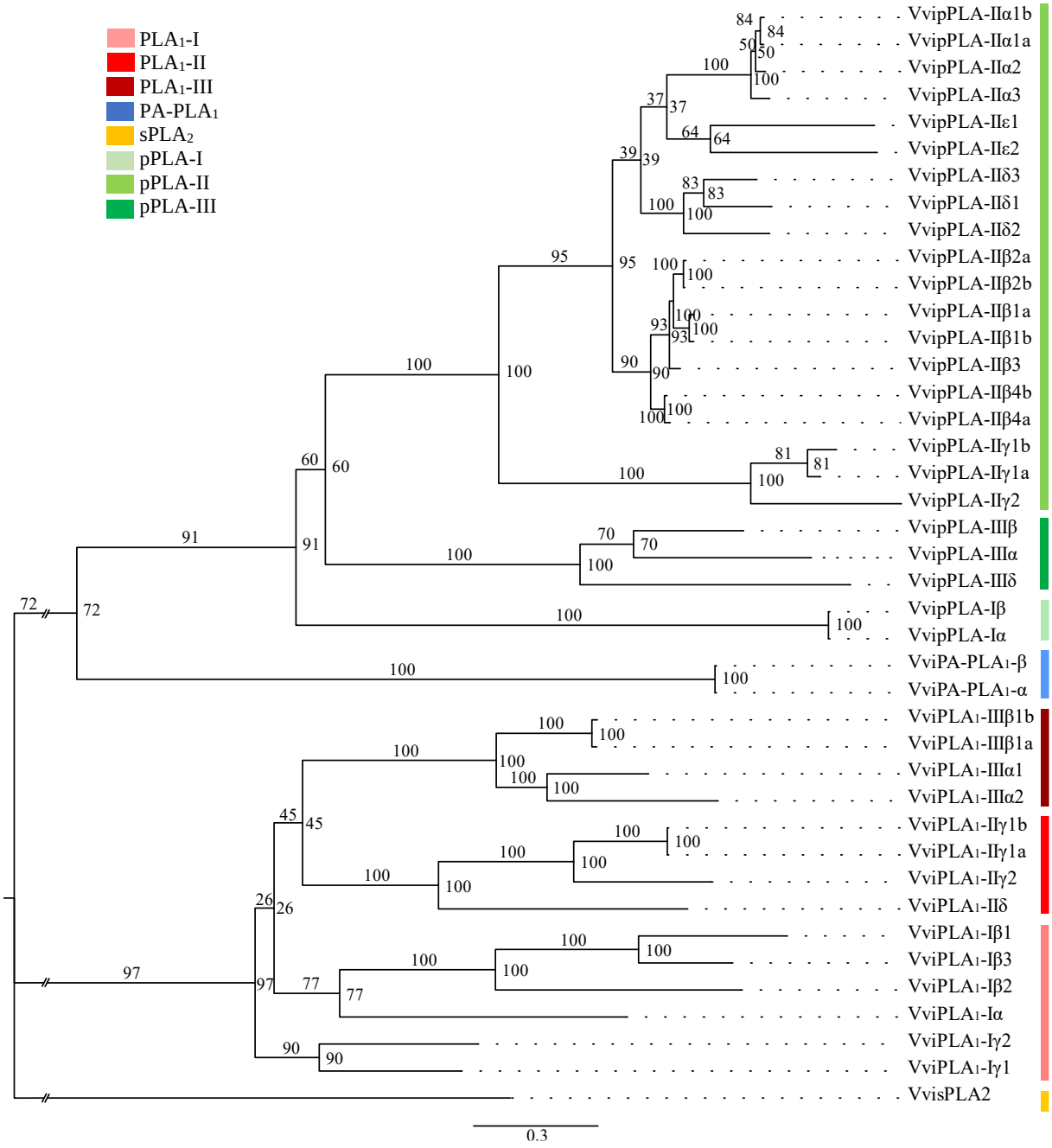

Figure 8 - Maximum likelihood phylogenetic tree of the *Vitis* PLA superfamily.
The numbers above branches show bootstrap values. Scale bar represents the number of estimated changes per branch length. Root was truncated with double dash totalling 0.3 changes per branch length.

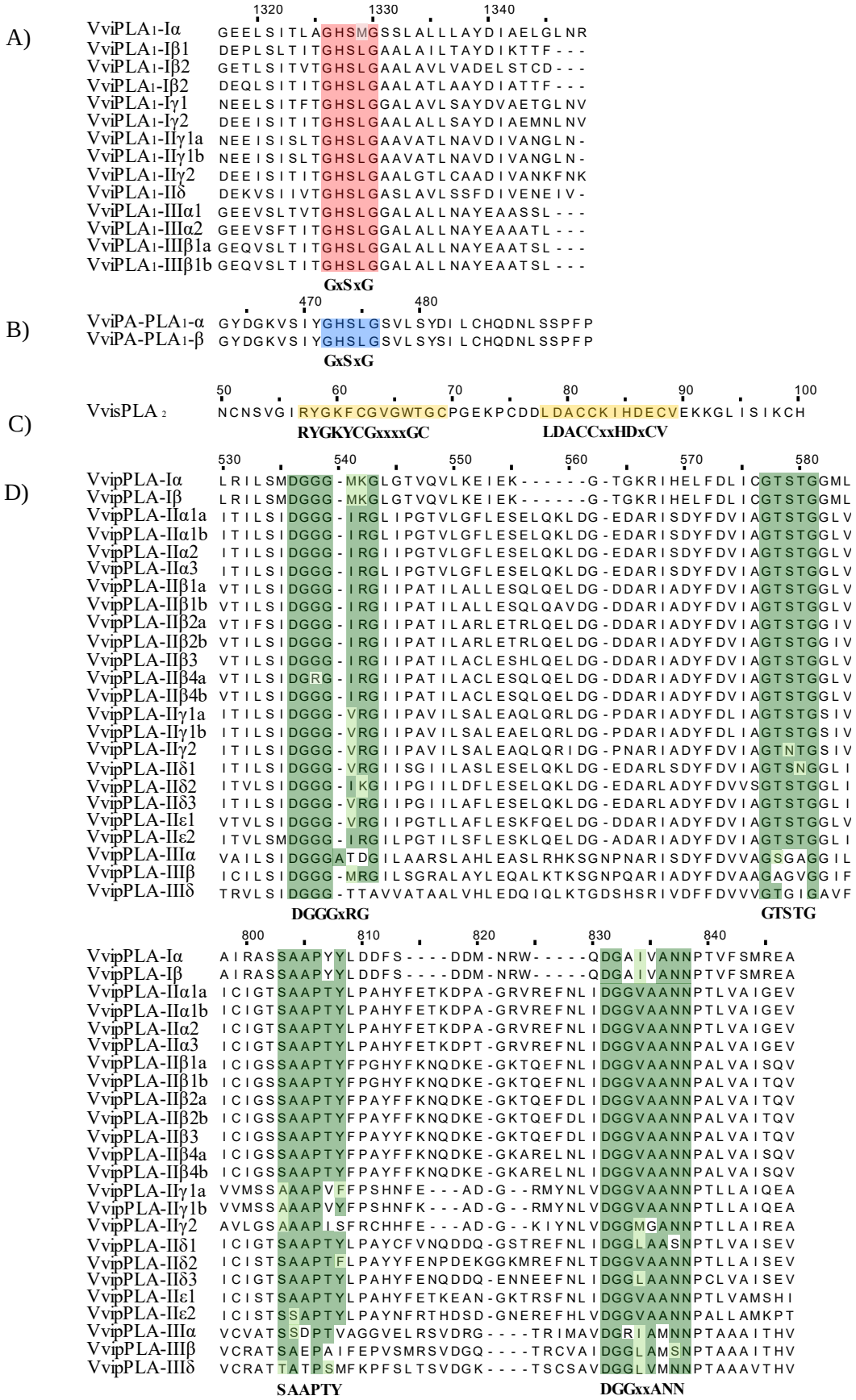

Figure 9 - Multiple alignment of four PLA families representing the consensus and conserved motifs.
Protein sequences were aligned for each PLA family, separately, applying MAFFT tool. The consensus motifs have been shown in shadow boxes according BLOSUM62. A) VviDAD-PLA$_1$; B) VviPA-PLA$_1$; C) VviPLA$_2$; D) VvipPLA

3.7. Selection of PLA for expression analysis

In plants, the activity of PLA has been described in several stressful conditions through the releasing of FA, by hydrolysis. Free FA will serve as substrate for the biosynthesis of signalling molecules, as JA. We have selected some PLA genes, putatively involved in grapevine immunity, for expression analysis by qPCR, namely *VviPLA1-Iβ1, VviPLA1-Iγ1* and *VviPLA1-IIδ* (groups I and II of DAD-PLA_1); *VvisPLA*$_2$ (sPLA_2) and *VvipPLA-I*, *VvipPLA-IIβ*, *VvipPLA-IIδ2* and *VvipPLA-IIIβ* (pPLA).

These specific genes were selected based on the homology which present with *Arabidopsis* PLA genes involved in JA production, defence response and galactolipids metabolism. Also, the chromosomal proximity to several grapevine chromosomal locus associated with the resistance to *P. viticola*, named RPV´s[79–87,124], was taking into account for the PLA gene selection (Supplementary data 4).

3.8. Expression analysis

Until now, our results suggest that the resistant grapevine cultivar present the most significant changes in lipid and FA content, upon infection with *P. viticola*, demonstrates that this genotype may have some mechanisms to trigger immunity that the susceptible does not possess. Taking this into account, the gene expression profiles of the selected grapevine PLA were analysed in *V. vinifera* cv. Regent at 6, 12 and 24 hours after inoculation with *P. viticola*.

The role of PLA_1 in JA biosynthesis has been elucidated in the last years[22,125]. The selected grapevine PLA_1 *VviPLA1-Iβ1* and *VviPLA1-Iγ1* present sequence homology with *Arabidopsis AtPLA*$_1$*Iβ1* and *AtPLA*$_1$*Iγ2*, respectively. that encodes to chloroplastidial PLA_1 involved in JA biosynthesis[125]. The expression of both *VviPLA1-Iβ1* and *VviPLA1-Iγ1* genes was incremented after *P. viticola* inoculation at all time-points, highlighting the high increase of *VviPLA1-Iβ1* at 12 hpi (114.87±42.36), (Figure 10). The *VviPLA1-IIδ* (presenting sequence identity with *Arabidopsis AtPLA*$_1$*IIδ*) showed an up-regulated only at 6 hpi. In *Arabidopsis*, *AtPLA*$_1$*IIδ* encodes for a PLA_1 located in both chloroplast and cytoplasm, that presents a catabolic function and participate in leaf senescence[126]. Allied to the fact of these genes encodes proteins located in chloroplast and have hydrolytic activity, these PLA_1 proteins may be responsible for FA release from galactolipids MGDG and DGDG, providing the biosynthetic precursors of JA and AzA.

In the pPLA family we have selected genes from all the three groups, being that only the members of groups I and II was its expression up-regulated (Figure 10). One of them was the *VvipPLA-I* that its *Arabidopsis* homologous encodes a large protein located in cytosol and chloroplast[44]. This increase may be related with an enhanced protein activity culminating in FA release from galactolipids, by it turns are the JA precursor.

Within the selected genes from the pPLA family, we have to highlight the gene expression of *VvipPLA-IIβ* that was very increased in Regent at 12 and 24 hpi after inoculation with *P. viticola* (Figure 10). Its *Arabidopsis* homologous, *AtpPLA-IIβ* catalysis the FA hydrolysis from galactolipids, PG, PC and PI[127]. Interesting it is the gene with higher fold-change, at 24 hpi (Figure 10). Its protein activity seems to act in cytosol (Supplementary data 2) and may be one of the responsible for the free FA release from PC, PE, PI and PA. The patatin-like PLA genes *VvipPLA-I* and *VvipPLA-IIδ2* (located near to RPV9) showed a slight expression increase at all time-points (Figure 10). At last, the *VvipPLA-IIIβ* gene was down-regulated at 6 and 12 hpi, and at 24 hpi its expression was not altered by the *P. viticola* inoculation (Figure 10). Its coding protein presents homology to *AtpPLA-IIIβ* that encodes a pPLA involved in lipid metabolic process[128], apparently with no role in grapevine response do *P. viticola*.

Another analysed PLA was the *VvisPLA*$_2$, which the *Arabidopsis* homologous, *AtsPLA*$_2$ encodes a group of small proteins with elevated activity in response to pathogen elicitor[129]. Secretory PLA_2 act in several plant tissues and could trigger responses to stressful conditions such as JA production, H^+-ATPase stimulation, and stomata opening[129,130]. Under pathogen attack the VvisPLA_2 may participate in FA releasing from membrane, one of them the C18:3 providing substrate for JA biosynthesis. One of the

VvisPLA$_2$ target could be the cell guard, in stoma. In *Arabidopsis* was observed that the AtsPLA$_2$-*β* act in cell guard and participates in stoma opening under light condition[130]. Once *P. viticola* infects the leaf by stoma[131] the expression increment of *VvisPLA$_2$* (Figure 10) suggest that this PLA may act in guard cell defence mechanism in the early hours of interaction between grapevine and *P. viticola*, preventing the progression of infection regulating stoma opening. These hypothesis gains strength by the slight increment in *VvisPLA$_2$* gene expression at 6 hpi (Figure 10).

Our results showed that in the resistant genotype, the gene expression of several PLA is increased under *P. viticola* inoculation that may suggest an involvement in the strong and quick plant defence response. This up-regulation of PLA at first hour post-inoculation is in concordance with our lipid analysis. We detected a lipid and FA modulation in the resistant grapevine upon infection that suggested its involvement in an efficient plant defence response. Both galactolipids and C18:3 presented the greatest modulation (Figure 5A; Figure 6A, B). Its participation in oxylipins formation, such as JA, has been highlighted, as well as the PLA participation in FA released from lipids membranes. These results reinforce our previous studies where was demonstrated an abrupt JA production at the first hours after infection with *P. viticola*[62,63].

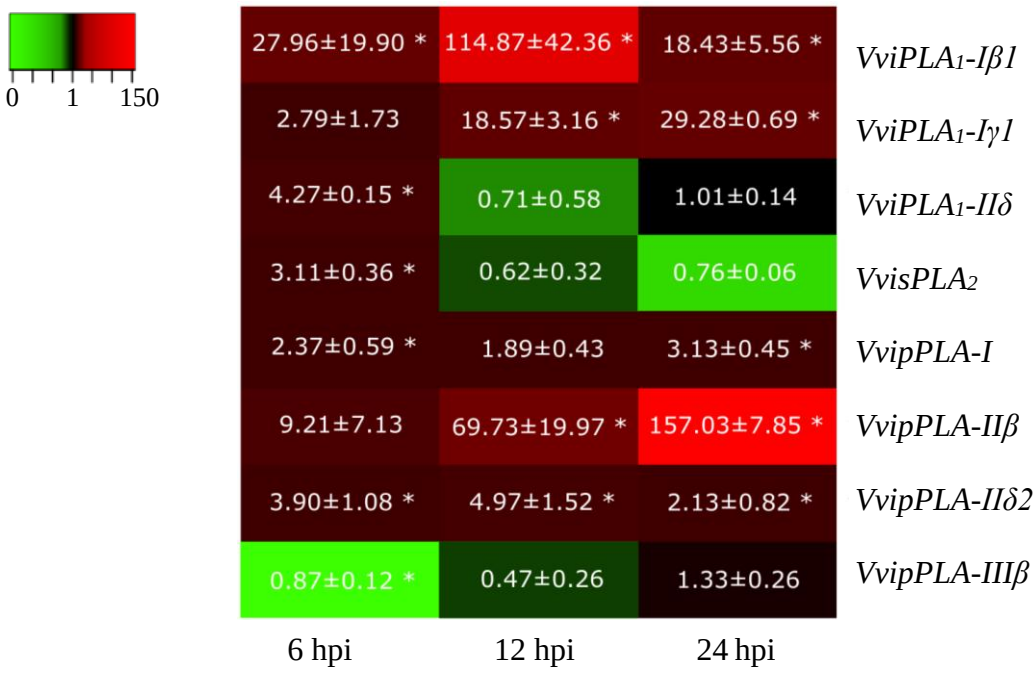

Figure 10 - Gene expression profile in Regent inoculated leaves.
For each time point (6, 12 and 24 hpi) gene transcripts fold-change relative to controls are represented for *VviPLA$_1$-Iβ1*; *VviPLA$_1$-Iγ1*; *VviPLA$_1$-IIδ*; *VvisPLA$_2$*; *VvipPLA-I*; *VvipPLA-IIβ*; *VvipPLA-IIδ2*; *VvipPLA-IIIβ*. Fold-change values are relative to expression in mock leaves. (*) Represents the statistically different between inoculated and control samples ($p<0.05$).

3.9. FA variation in grapevine species – biomarker establishment

In our previous results, we have shown that both lipid and FA content allowed discrimination the two grapevine genotypes, Trincadeira and Regent, prior to *P. viticola* inoculation. We have analysed FA and lipid content a several grapevine accessions (*Vitis* species and *V. vinifera* cultivars) with different degree of tolerance to *P. viticola*, prior to pathogen inoculation in order to understand if FA may be used as biomarkers.

The *Vitis* species and *Vitis vinifera* cultivars were divided in two groups. The susceptible group include *V. vinifera* cv. Pinot noir, Trincadeira and Cabernet Sauvignon and the resistant group (comprising tolerant and resistant grapevines to *P. viticola*) include *V. Riparia*, *V. Labrusca*, *V. Rupestris*, *V. Rotundifolia*, *V. Candicans*, *V. Sylvesteris* and *V. vinifera* cv. Regent.

Principal component analysis (PCA) and similarity percentages (SIMPER) revealed distinctive differences between susceptible and resistant grapevines. PCA score plot showed the two groups are mainly separated along PC1 (68.2%), being C18:3 the FA responsible for the separation, probably due the high abundancy presents in these samples (Figure 11). PC2 (25.3%) is visible a tendency for separation between susceptible and resistant *Vitis*, mainly contributed by C16:0 and C18:2 for the separation between resistant group (Figure 11). SIMPER analysis showed that C18:3 is the FA that mostly contribute for the aggregation of both susceptible and resistant groups as well as the dissociation between these groups.

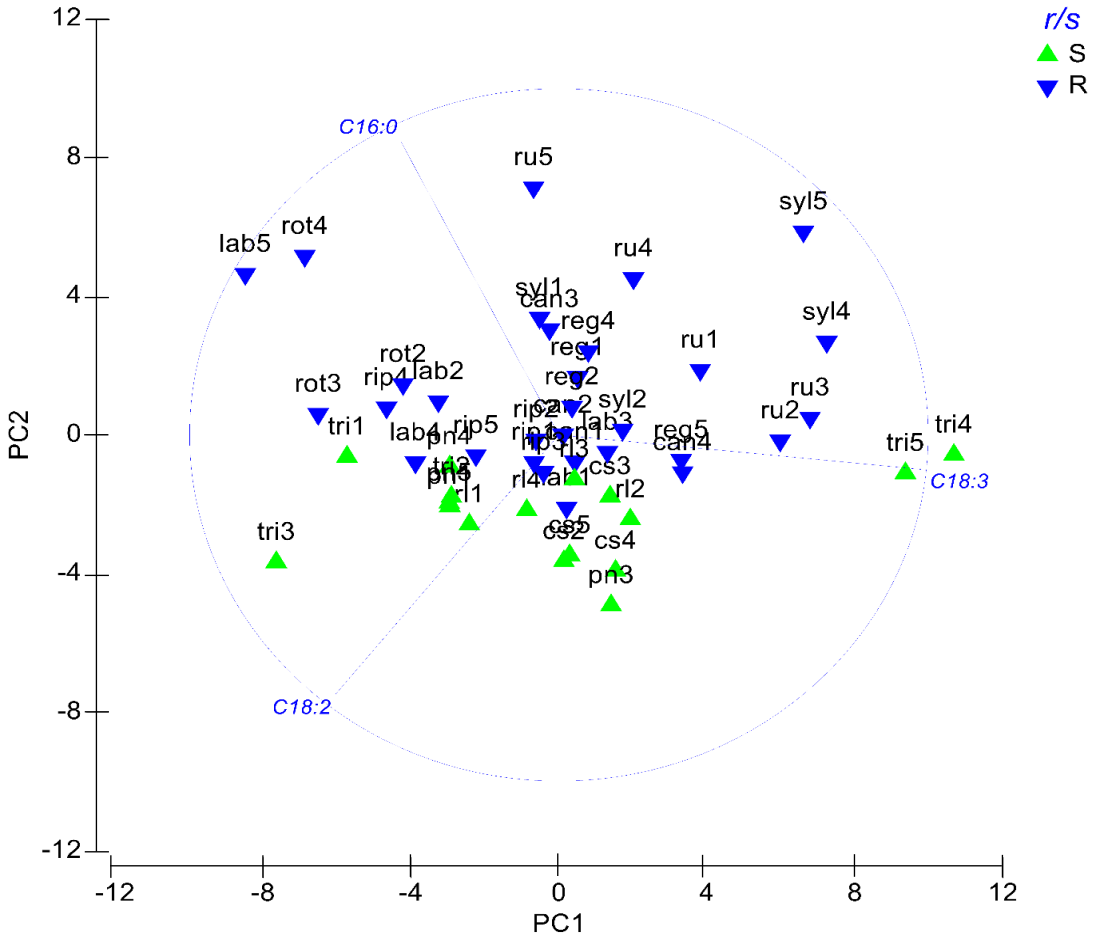

Figure 11 – Principal component analysis (PCA) diagram of the total of fatty acids content from susceptible (▲) and resistant (▼) grapevines leaves to *Plasmopara viticola*.
Score plots of susceptible (*V. vinifera* cv. Pinot noir, Trincadeira and Cabernet Sauvignon) and resistant grapevines (*V. Riparia*, *V. Labrusca*, *V. Rupestris*, *V. Rotundifolia*, *V. Candicans*, *V. Sylvesteris* and *V. vinifera* cv. Regent).
PC % variations: PC1=62.8, PC2=25.3 of 5 biological samples. Fatty acids correlation ≥ 0.9.

We have also selected two genotypes: *Vitis riparia* (resistant to *P. viticola*) and *V. vinifera* cv. Pinot noir (susceptible) and characterized their basal lipid content by TLC.
The resistant *Vitis* specie presented a lower content of MGDG, a higher content of PG and no significant differences in the DGDG (Figure 12a), resulting in a lower galactoglycerolipids/phosphoglycerolipids ratio (Figure 12A, B). In the extraplastidial lipids, only the neutral lipids (NL) presented differences in its content, being higher in *V. riparia* (Figure 12A). The NL includes TAG and free FA in its content. When comparing these results to the previously described results for *V. vinifera* cv. Regent and cv Trincadeira, there is an evident similarity between the resistant genotypes. *V. vinifera* cv. Regent and *V. riparia* have a lower content in plastidial lipids, as result of the lower MGDG content, comparatively to *V. vinifera* cv. Trincadeira and *V. vinifera* cv. Pinot Noir.

Other similarities are found in PG and NL, being these lipids are present in higher content in the resistant grapevines. It should be noted that NL once contain free FA and TAG, the soma of these lipids in Regent is higher than in Trincadeira, culminating in a higher content of NL in resistant, like in *V. riparia* in relation with Pinot Noir.

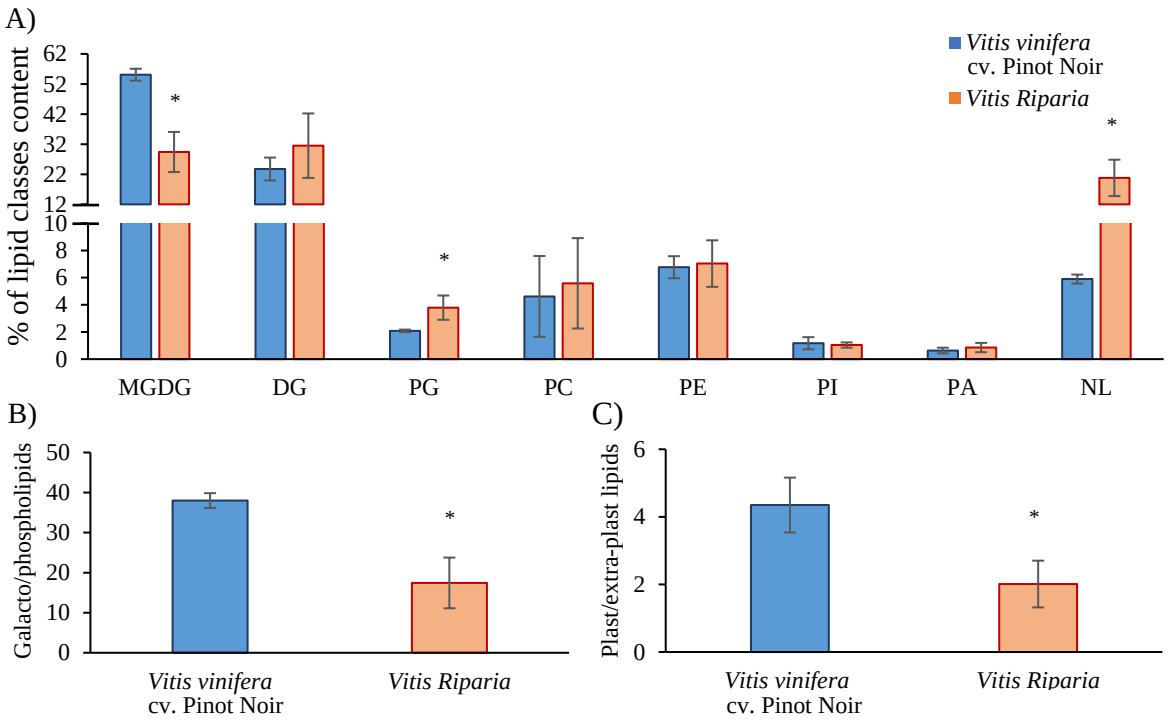

Figure 12 - Lipid composition of *Vitis vinifera* cv. Pinot noir and *Vitis riparia* leaves.
(A) Total of lipids content; B) ratio galactoglycerolipids/phosphoglycerolipids; C) plastidial lipids/extra-plastidial lipids. Asterisk marks (*) represents the statistically differences between *Vitis vinifera* cv. Pinot noir and *Vitis riparia* at $p<0.05$. Values correspond to average relative percentage ± standard error, n = 3.

The differences either in lipids or FA between susceptible and resistant/tolerant grapevines to *P. viticola* demonstrate an evident pattern. One of breeders' goals is the introgression of resistant traits, in order to create hybrid grapevines that are resistant to this pathogen while conserving fruit and wine properties. However, in grapevine breeding, the selection of plants that present the resistant trait is a long and laborious process.
This feature could be a useful for biomarkers creation, a tool that will allow distinguish *Vitis* genotypes with tolerance/resistant to the pathogen from the susceptible genotypes in an early stage of grapevine development, reducing the selection time of hybrid grapevines. More studies considering more species and varieties should be conducted in order to validate lipids and FA as biomarkers for breeding purposes.

4. Conclusion

Plant lipids participate in many biological processes but their capacity to modulate defence mechanisms as gained special attention. In the present work both lipid and FA profile of two *Vitis vinifera* cultivars, Regent and Trincadeira, leaves were discriminated. This lipidomic approach highlighted a distinct constitutive composition of lipid and FA of two grapevine genotypes, *V. vinifera* cv Regent and cv Trincadeira, resistant and susceptible to the downy mildew pathogen, being the main differences related to the plastidial lipids (MGDG, DGDG and PG), signalling lipids (PI and PA) and in free FA content.
In the first hours after pathogen challenge, a differential modulation of lipids is found, being more pronounced in the resistant genotype. After inoculation, the resistant genotype presents an alteration of several lipid classes, being galactolipids and free FA the most affected. Galactolipids were also the lipid class where major alterations in FA content, mainly PUFAs, were observed. In the susceptible genotype, lipid modulation upon pathogen inoculation was negligible, thus suggesting that this process is only activated in the resistant genotype and is related to the arrest of pathogen development, leading to the establishment of the incompatible interaction. In fact, one of the features of Regent's defence response against *P. viticola* is the activation of the JA biosynthesis and signalling pathways[62,63,132]. Linolenic acid (C18:3), one of the JA precursors, is the most abundant FA in galactolipids and presented an increase after pathogen challenge. Fatty acid hydrolysis is catalysed by PLA allowing FA release from membranes and PLA activity has been associated to several plant defence mechanisms[4,6,37,38,43], thus highlighting its importance in the establishment of an effective resistance response. Phospholipase A may play an important role in grapevine-*P. viticola* pathosystem. As this superfamily has yet not been characterized in grapevine, we used several bioinformatic tools to characterize its members. Forty-one PLA genes were identified variably dispersed among 9 of the 19 grapevine chromosomes. *Vitis vinifera* PLA genes encodes to proteins distributed in three major classes, according with their similarity. Phospholipase A proteins were predictably located, mostly, in cytosol and chloroplast. We have also selected several PLA genes, already described in other pathosystem or related to the RPV loci in grapevine to access their expression profile in the resistant genotype. Our results showed an increase of expression, in most of the selected PLA, being 6 hpi, the time-point were more relevant alterations occur. These evidences together with our lipidomic analysis and our previous results[62,63,132] suggest that the selected PLA probably are involved in grapevine defence response, by releasing FA from lipid membranes, generating free FA that will act directly, as signalling molecules, or indirectly serving as biosynthetic precursors of AzA or JA (Figure 13).

We have also discovered that both FA and lipid profile allowed discriminating both genotypes prior to inoculation, thus we evaluated FA composition of several grapevines genotypes, with different tolerance degrees to *P. viticola*. We have selected two *Vitis, V. vinifera* cv. Pinot Noir and *V. riparia*, to access their lipid composition and to compare with the previously obtained results for Trincadeira and Regent. Tolerant grapevines demonstrate an evident FA pattern discriminating them from the susceptible group.

In summary, our results provide new evidences on lipid role in grapevine-*P. viticola* pathosystem. Further studies on the involvement of other PLA and lipid processing enzymes such as desaturases are crucial for a deeper understanding of this process, moreover analysing other time-points of interaction between this pathogen and grapevine will aid to fully access lipid role. Also, lipid and FA composition of grapevine leaves seems to be a promising indicator of resistance to downy mildew, thus further studies including more grapevine accessions may allow establishing resistance biomarkers.

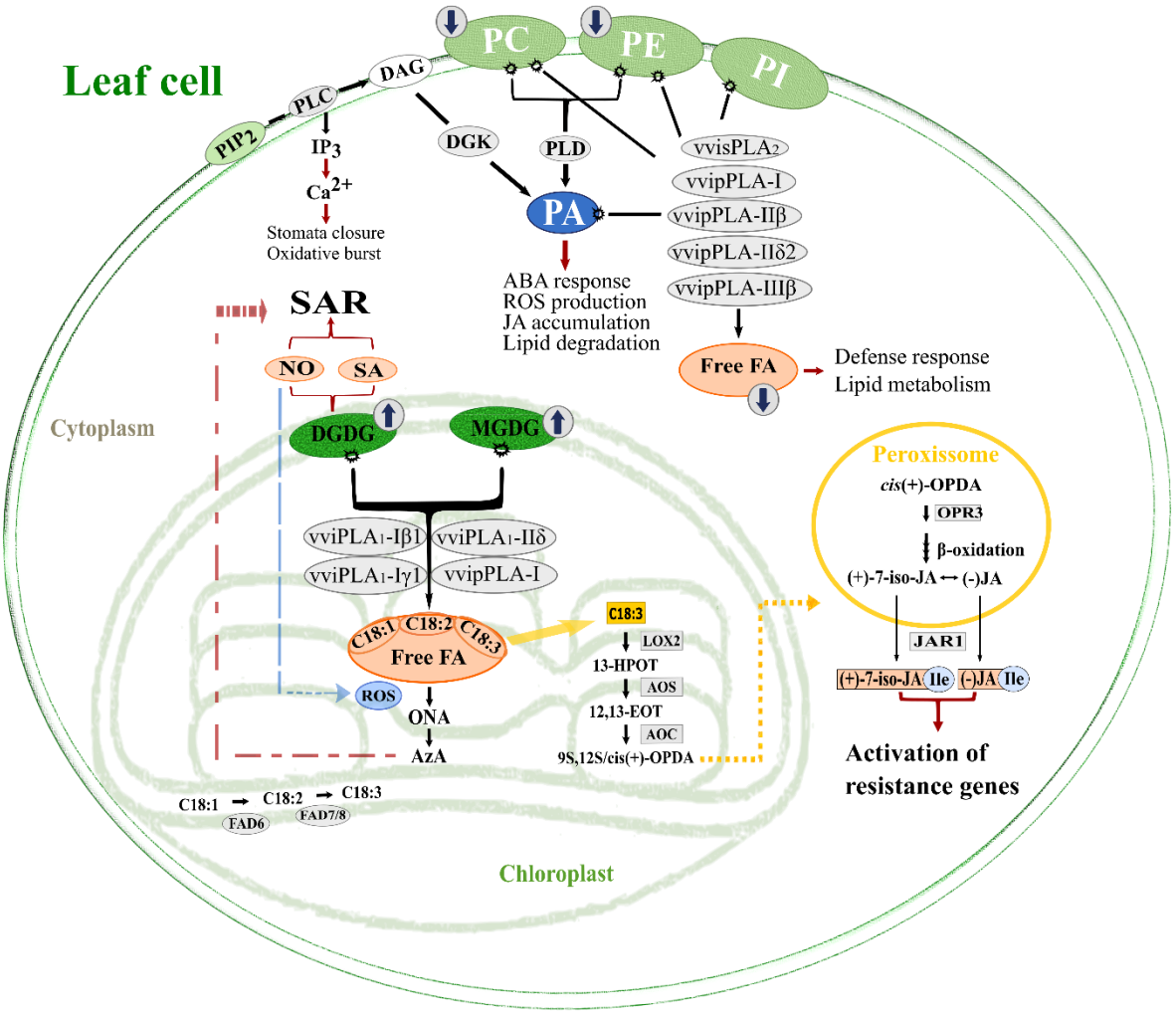

Figure 13 - PLA acting mechanisms of *Vitis vinifera* cv. Regent upon infection with *Plasmopara viticola*.

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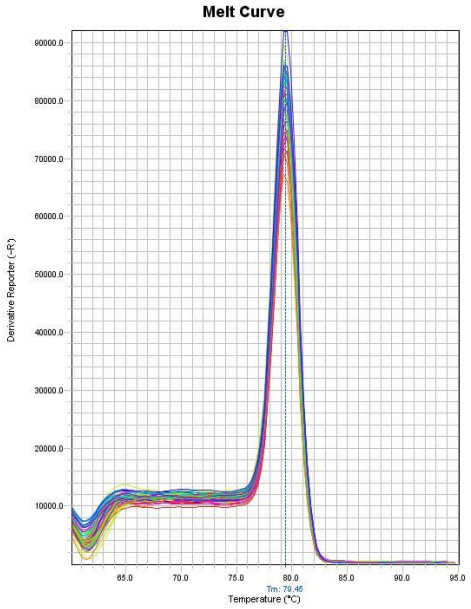
Appendix 1

A)



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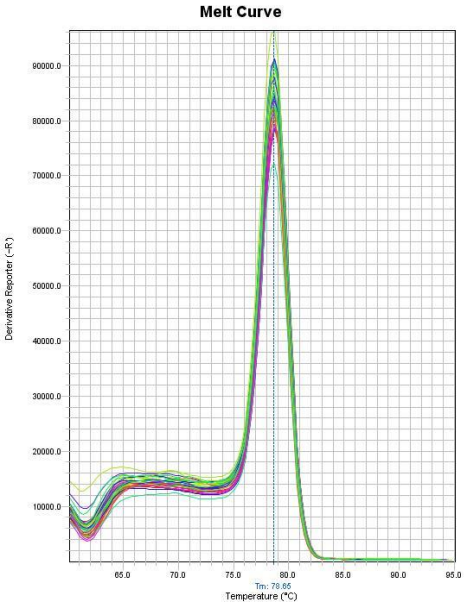

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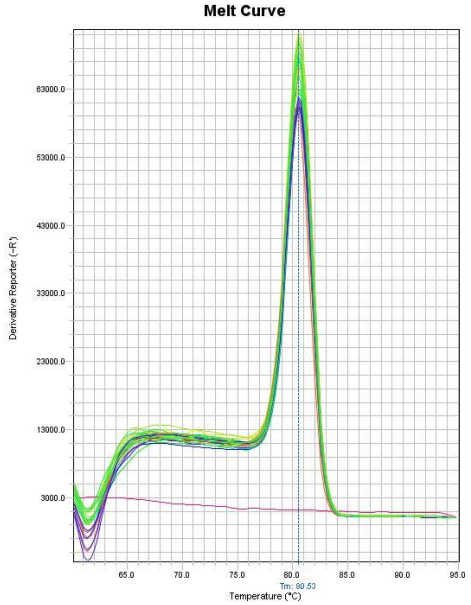

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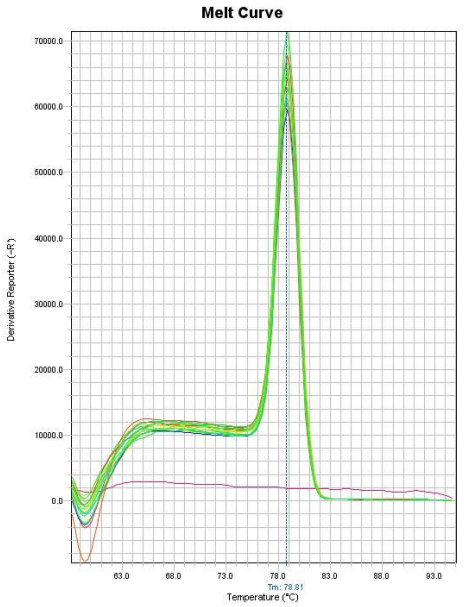

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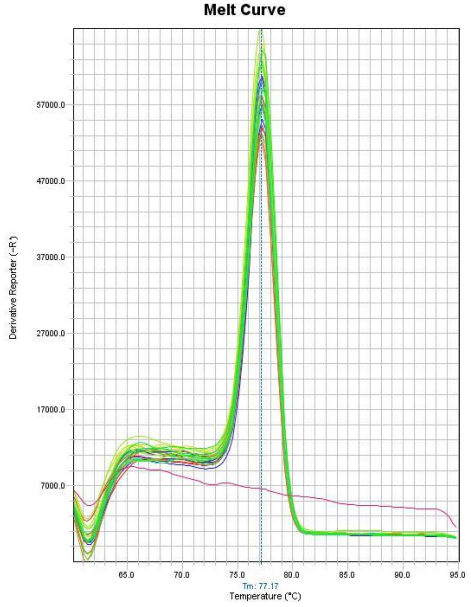

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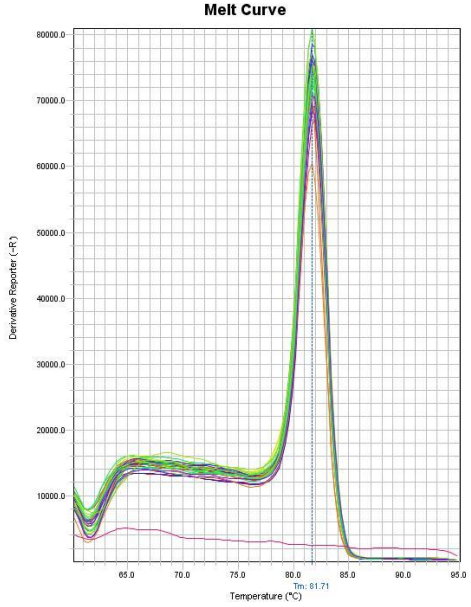

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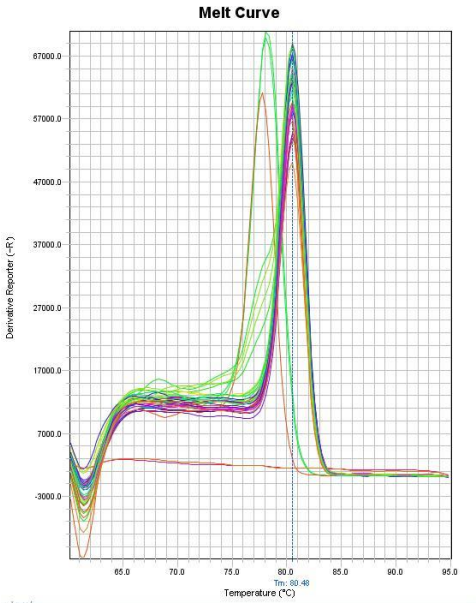

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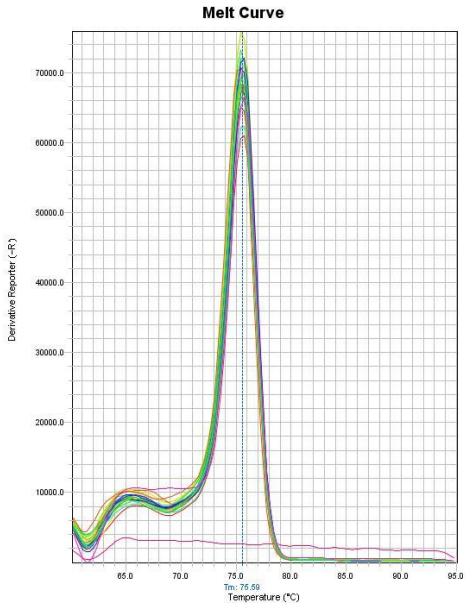

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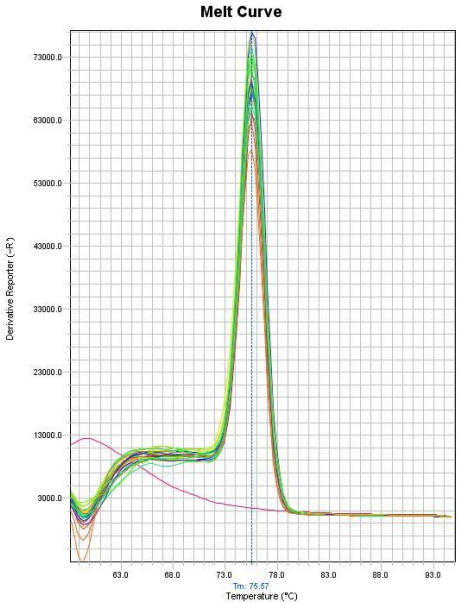

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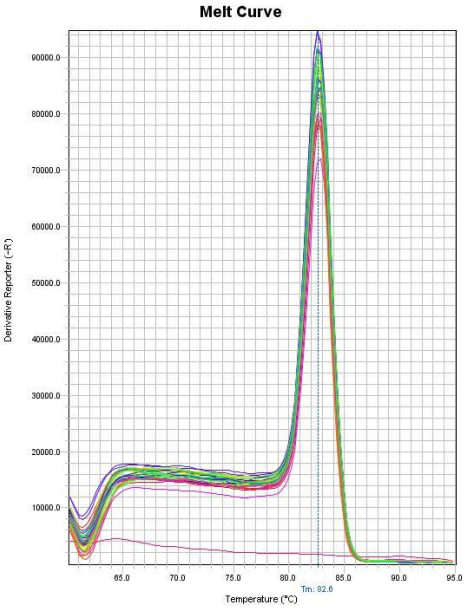


Supplementary data 1 - Melting curves of targeted genes.
A) *EF1α*; B) *UBQ*; C) *VviPLA₁-Iβ1*; D) *VviPLA₁-Iγ1*; E) *VviPLA₁-IIδ*; F) *VvisPLA₂*; G) *VvipPLA-I*; H) *VvipPLA-IIβ*; I) *VvipPLA-IIδ2*; J) *VvipPLA-III*.

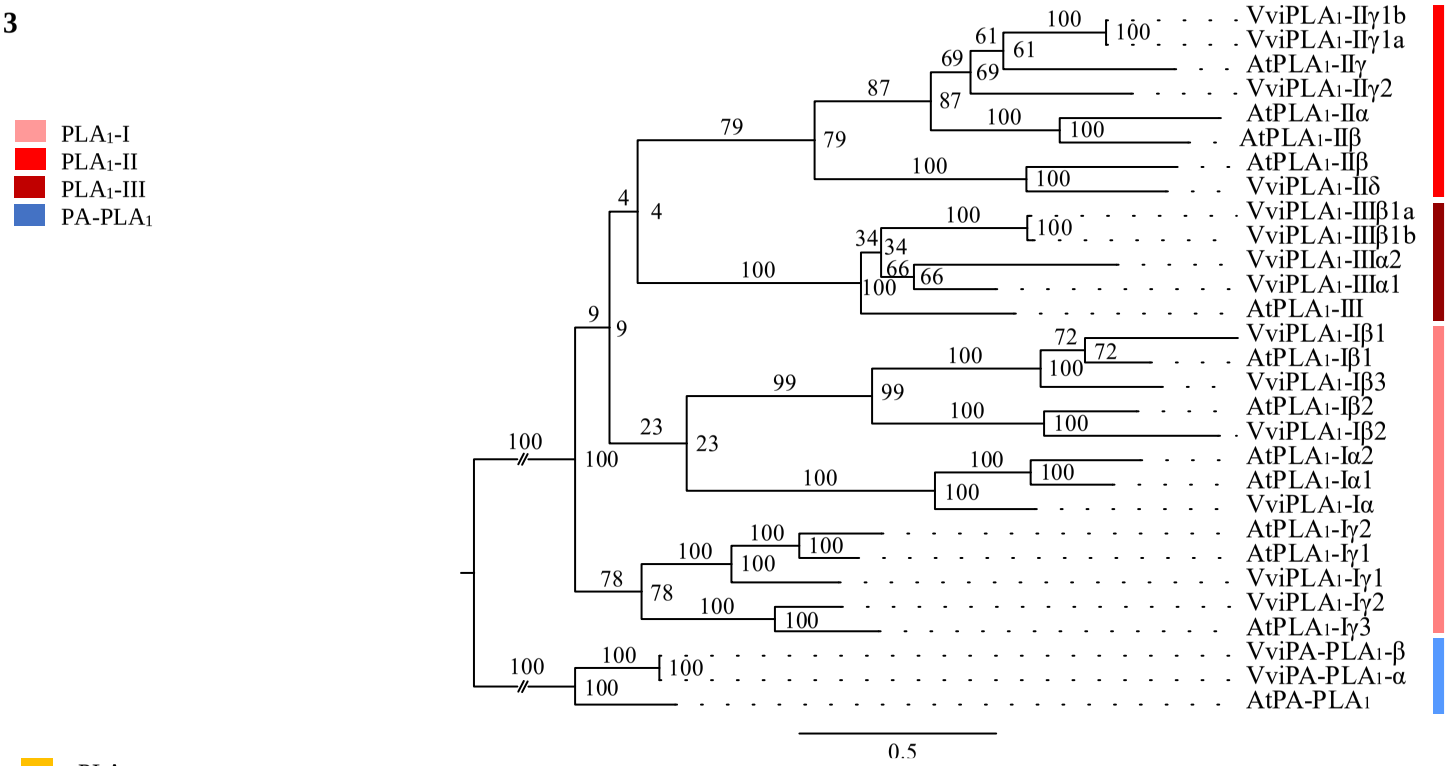
Appendix 2

Supplementary data 2 - Characterization of grapevine PLA superfamily table.
Proposed grapevine PLA nomenclature, gene locus, protein and nucleotide accessions (from NCBI and CRIBI), chromosome location, intron number, protein length, molecular weight, isoelectric point (pI), domain and subcellular prediction are represented.

Family	Group	Proposed nomenclature	Locus	Protein	Nucleotide	*Vitis* code	Chr.	Intron	Protein length (aa)	Molecular weight (kDa)	pI	Domain (Blast2GO)	Subcellular location
DAD PLA_1	**I**	VviPLA$_1$-Iα	LOC100242894	XP_002268142.1	XM_002268106.2	VIT_205s0124g00210.1	5	0	446	49.8	7.04	Lipase_3	Chloroplast
		VviPLA$_1$-Iβ1	LOC100242763	XP_002270992.2	XM_002270956.4	VIT_215s0021g01510.1	15	0	434	48.7	9.48	Lipase_3	Chloroplast
		VviPLA$_1$-Iβ2	LOC100247003	XP_010647255.1	XM_010648953.2	VIT_200s0620g00010.1	unknown	0	528	59.1	9.04	Lipase_3	Chloroplast
		VviPLA$_1$-Iβ3	LOC100259863	XP_002271065.3	XM_002271029.4	VIT_215s0021g01520.1	15	0	389	43.3	8.82	Lipase_3	-
		VviPLA$_1$-Iγ1	LOC100249474	XP_002281907.1	XM_002281871.3	VIT_207s0141g00360.1	7	1	514	58.2	7.07	Lipase_3	Chloroplast
		VviPLA$_1$-Iγ2	LOC100260417	XP_002285367.1	XM_002285331.3	VIT_206s0004g02390.1	6	0	502	57.7	8.59	Lipase_3	Chloroplast
	II	VviPLA$_1$-IIγ1a	LOC100242927	XP_002266982.2	XM_002266946.3	VIT_210s0003g01040.1	10	2	369	41.2	6.73	Lipase_3	Mitochondrial
		VviPLA$_1$-IIγ1b	LOC100242927	XP_019078036.1	XM_019222491.1	VIT_210s0003g01040.1	10	2	406	45.5	6.73	Lipase_3	Mitochondrial
		VviPLA$_1$-IIγ2	LOC100254098	XP_019079306.1	XM_019223761.1	VIT_212s0028g02470.1	12	3	403	45.7	8.93	Lipase_3	Mitochondrial
		VviPLA$_1$-IIδ	LOC100267811	XP_002281095.2	XM_002281059.4	VIT_212s0028g02000.1	12	0	470	53.1	5.84	Lipase_3	Chloroplast
	III	VviPLA$_1$-IIIα1	LOC100266852	XP_002272780.2	XM_002272744.3	VIT_210s0003g04200.1	10	0	524	59.7	9.51	Lipase_3	-
		VviPLA$_1$-IIIα2	LOC100242997	XP_002272558.2	XM_002272522.4	VIT_210s0003g04200.1	10	0	511	58.8	7.32	Lipase_3	-
		VviPLA$_1$-IIIβ1a	LOC100251427	XP_010655698.1	XM_010657396.2	VIT_210s0003g04230.1	10	0	510	58.0	7.07	Lipase_3	Chloroplast
		VviPLA$_1$-IIIβ1b	LOC100261664	XP_010655697.1	XM_010657395.2	VIT_210s0003g04210.1	10	0	510	58.1	7.72	Lipase_3	Chloroplast
PA-preferring PLA_1		VviPA-PLA$_1$-α	LOC100260107	XP_002275612.1	XM_002275576.3	VIT_218s0082g00108.1	18	20	971	109.4	5.03	DDHD	vacuole membrane / plasmodesma
		VviPA-PLA$_1$-β	LOC100260107	XP_010645877.1	XM_010647575.2	VIT_218s0082g00108.1	18	20	963	108.5	5.02	DDHD	vacuole membrane / plasmodesma
Secretory PLA2		VvisPLA$_2$	LOC100261227	XP_002282822.1	XM_002282786.3	VIT_211s0016g02570.2	11	3	151	16.3	8.70	-	integral component of membrane
Patatin-like PLA	**I**	VvipPLA-Iα	LOC104880454	XP_010655361.1	XM_010657059.2	VIT_210s0116g00830.4	10	18	1067	118.5	5.88	Arm / Patatin domain	-
		VvipPLA-Iβ	LOC104880454	XP_019078143.1	XM_019222598.1 XM_010657058.2	VIT_210s0116g00830.1 VIT_210s0116g00830.1	10	18	1316	146.2	5.83	Arm / Patatin domain	-
	II	VvipPLA-IIα1a	LOC100257463	XP_002282481.1	XM_002282445.3	VIT_218s0001g10910.1	18	6	413	45.0	5.97	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIα1b	LOC109121419	XP_010664584.1	XM_010666282.2	VIT_218s0001g10870.1	18	6	413	45.1	5.97	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIα2	LOC100266120	XP_002282597.1	XM_002282561.3	VIT_218s0001g10910.1	18	6	413	45.0	5.81	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIα3	LOC100241962	XP_002284571.1	XM_002284535.4	VIT_218s0001g10830.1	18	6	425	46.5	5.28	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIβ1a	LOC100252227	XP_002282366.2	XM_002282330.3	VIT_218s0001g11010.1	18	6	411	45.4	6.35	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIβ1b	LOC100264244	XP_019071995.1	XM_019216450.1	VIT_218s0001g11010.1	18	6	411	45.3	6.35	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIβ2a	LOC100248840	XP_002282415.2	XM_002282379.2	VIT_218s0001g10970.1	18	6	406	44.9	6.98	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIβ2b	LOC100260810	XP_002282432.1	XM_002282396.2	VIT_218s0001g10950.1	18	7	406	44.9	6.98	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIβ3	LOC100255738	XP_002282440.1	XM_002282404.1	VIT_218s0001g10940.1	18	6	406	44.9	5.95	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIβ4a	LOC100254055	XP_019071859.1	XM_019216314.1	VIT_218s0001g10880.1	18	7	406	44.8	8.86	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIβ4b	LOC100267749	XP_019071978.1	XM_019216433.1	VIT_218s0001g10920.1	18	6	406	44.7	8.85	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIγ1a	LOC100260531	XP_010652290.1	XM_010653988.2	VIT_207s0005g01860.1	7	12	425	47.0	6.27	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIγ1b	LOC100264086	XP_002272043.1	XM_002272007.3	VIT_207s0005g01890.1	7	6	426	47.1	6.33	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIγ2	LOC100260531	XP_002271702.1	XM_002271666.3	VIT_207s0005g01840.1	7	12	424	46.6	9.21	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIδ1	LOC100249295	XP_002281798.1	XM_002281762.4	VIT_207s0031g00870.1	7	6	407	4.40	5.31	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIδ2	LOC100245674	XP_002277318.1	XM_002277282.3	VIT_207s0031g00850.2	7	6	402	44.2	5.48	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIδ3	LOC100250815	XP_002277305.1	XM_002277269.2	VIT_207s0031g00850.1	7	7	407	44.5	5.86	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIε1	LOC100244172	XP_002262942.1	XM_002262906.3	VIT_200s0567g00060.1	unknown	5	397	43.6	5.96	Patatin domain	Cytoplasm / membrane
		VvipPLA-IIε2	LOC100262840	XP_019076557.1	XM_019221012.1	VIT_207s0031g00830.1	7	6	398	44.1	7.16	Patatin domain	Cytoplasm / membrane
	III	VvipPLA-IIIα	LOC100252820	XP_002276337.2	XM_002276301.3	VIT_210s0092g00150.1	10	1	484	52.4	9.18	Patatin domain	-
		VvipPLA-IIIβ	LOC100246118	XP_010658410.1	XM_010660108.2 XM_019224348.1	VIT_213s0019g04200.3 VIT_213s0019g04200.5	13	4	458	49.7	6.91	Patatin domain	-
		VvipPLA-IIIδ	LOC100263127	XP_002277881.1	XM_002277845.4	VIT_207s0151g01130.1	7	1	385	42.0	8.85	Patatin domain	-

Appendix 3

A)



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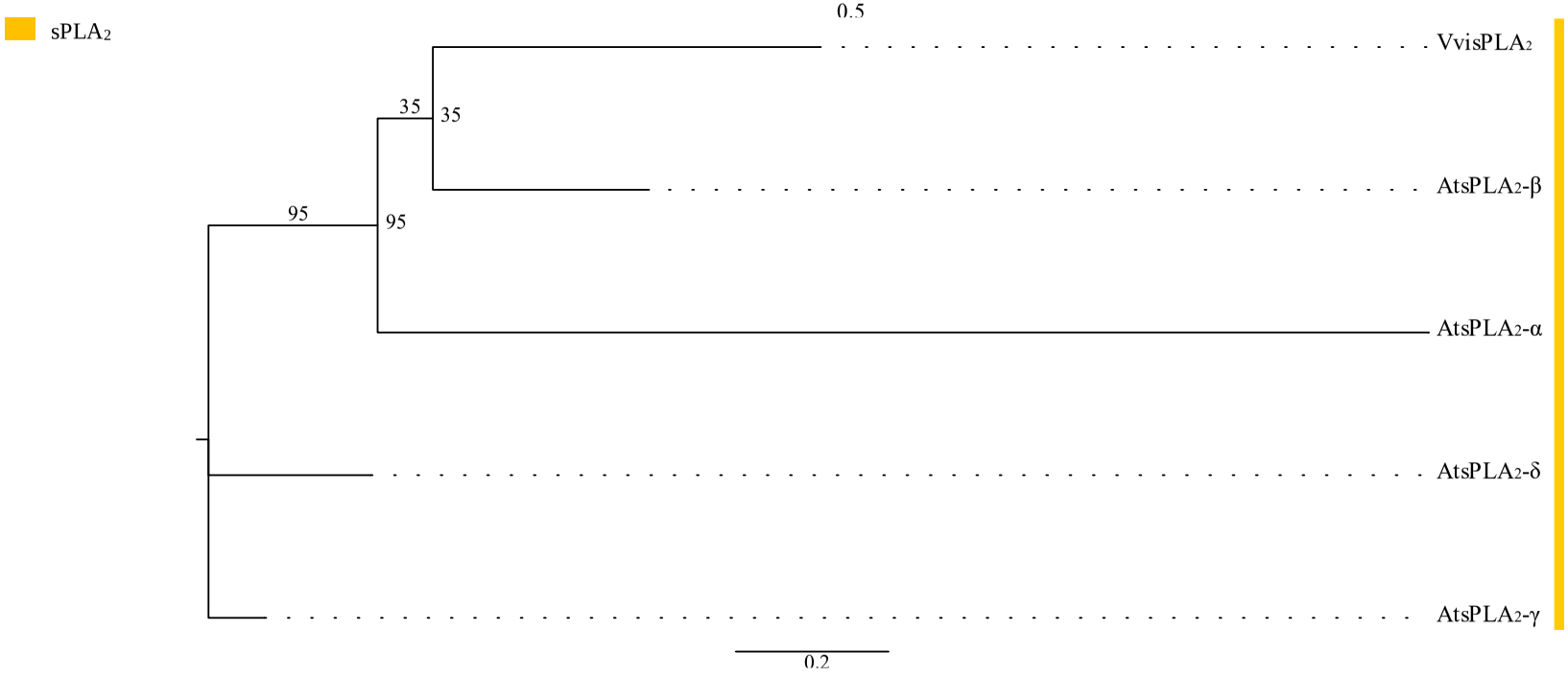

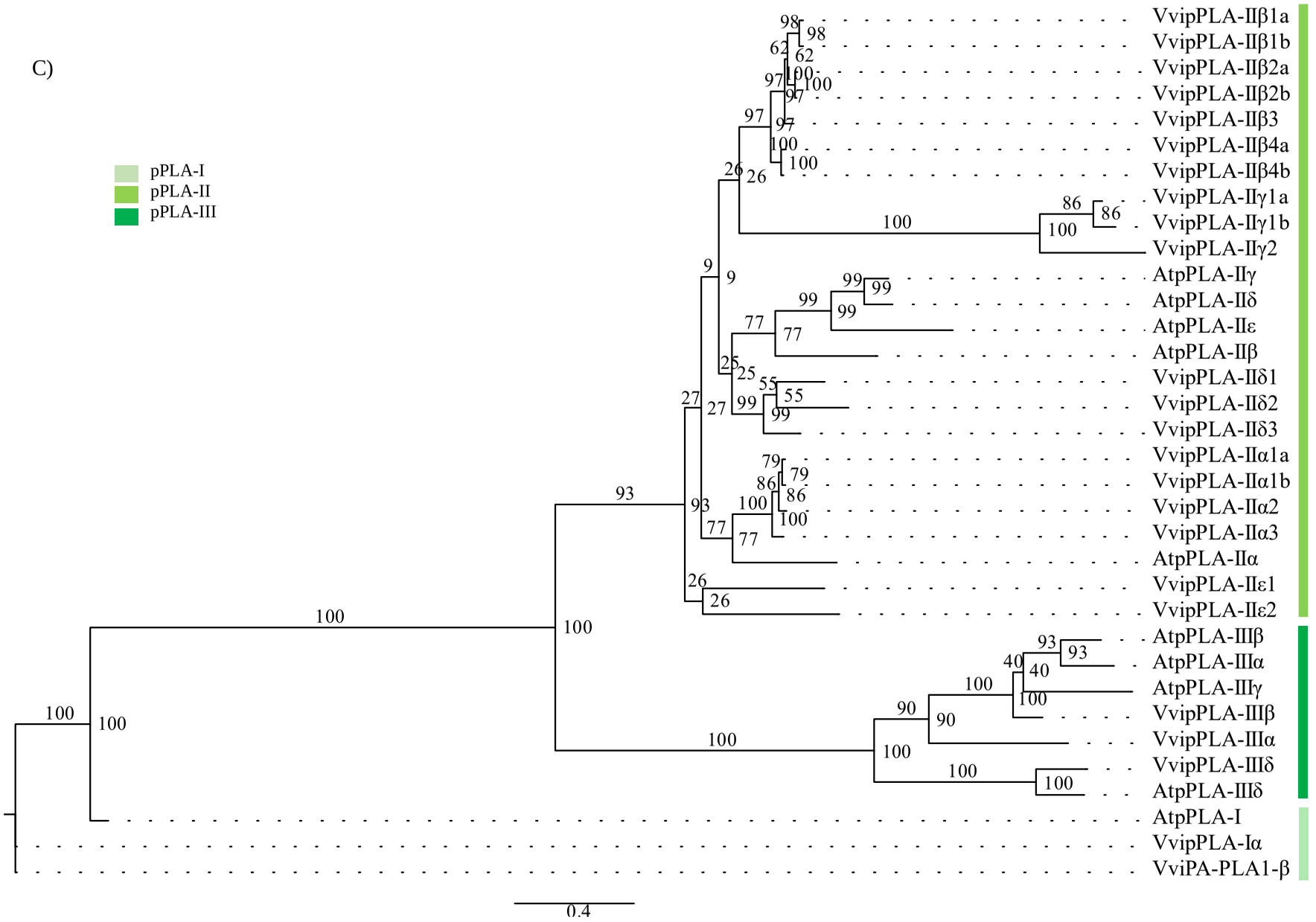

Supplementary data 3 - Maximum likelihood phylogenetic tree of the 41 from *Vitis* PLA proteins and 27 PLA proteins from A*rabidopsis thaliana*.
The phylogenetics trees are divided in classes: A) PLA_1, B) $sPLA_2$ and C) pPLA. The numbers above branches show bootstrap values. Scale bar represents the number of estimated changes per branch length. In A) the root was truncated with double dash totalling 1.5 changes per branch length.

Appendix 4

Supplementary data 4 - Grapevine PLA´s genes chromosomal location and chromosomal location of "Resistance to *Plasmopara viticola* (RPV)" loci.
Bold indicate PLA´s selected for further studies, bold and asterisks (*) indicate the PLA´s with putative involvement in defense.

PLA gene	Nucleotide	PLA Location (Mb)	Chr.	RPV	RPV Location (Mb)	Associated marker	References
VviPLA1-Iα	XM_002268106.2	20.6	5	RPV14		GF05-13	Ochssner et al. 2016
VviPLA$_1$-Iα2	XM_002281871.3	0.2					
VviPLA1-Iγ1	XM_002277845.4	1.3		RPV7	11.4	UDV-097	Bellin et al. 2009
VvipPLA-IIδ3*	XM_002277269.2	17					
VvipPLA-IIδ2*	XM_002277282.3	17	7				
VvipPLA-IIδ1*	XM_002281762.4	17				CCoAOMT	Moreira et al. 2011
VvipPLA-IIγ1a	XM_010653988.2	4.3		RPV9	16.6		
VvipPLA-IIγ1b	XM_002272007.3	4.3					
VviPLA1-IIδ	XM_002281059.4	2.6		RPV1	10.3	VVIb32	Merdinoglu et al. 2003
				RPV6	20.4	VMC8G9	Marguerit et al. 2009
VviPLA1-IIγ2	XM_019223761.1	3.1	12	RPV13	10.0		Moreira et al. 2011
VviPA-PLA1-α	XM_002275576.3	0.9		RPV2			Wiedemann-Merdinoglu et al. 2006; Bellin et al. 2009
VviPA-PLA1-β	XM_010647575.2	0.9					
vvipPLA-IIα3	XM_002284535.4	9.1					
VvipPLA-IIα2	XM_002282561.3	9.1			24.9	UDV-112	
				RPV3	26.9	UDV-305	Welter et al. 2007; Bellin et al. 2009
VvipPLA-IIα1b	XM_010666282.2	9.1				VMC7f2	
*VvipPLA-IIβ4a**	XM_019216314.1	9.1	18				
				RPV15			
VvipPLA-IIα1a	XM_002282445.3	9.2					Pap et al. (in preparation)
VvipPLA-IIβ3	XM_002282404.1	9.2					
VvipPLA-IIβ2b	XM_002282396.2	9.2					
VvipPLA-IIβ2a	XM_002282379.2	9.2					
VvipPLA-IIβ1b	XM_019216450.1	9.3					
VvipPLA-IIβ1a	XM_002282330.3	9.3					